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FILE 'MEDLINE, EMBASE, CAPLUS, BIOSIS' ENTERED AT 15:26:22 ON 12 NOV 1999
L1 6777 S BAX
L2 515803 S PLASMID# OR VECTOR#
L3 338 S L1 AND L2
L4 149 DUP REM L3 (189 DUPLICATES REMOVED)
L5 94 S L4 AND PY<1999
L6 83 S L5 AND APOPTOSIS
L7 2574859 S TUMOR# OR CANCER# OR NEOPLASTIC
L8 52 S L7 AND L6
L9 10 S L8 AND ADENOVIR?

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○	S4	unique items	All subject words	111
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S16		USPT	sensitizing or killing tumor cells and radiation or chemotherapy
S15		USPT	treatment or delivery or administration and (bax pro-apoptotic gene and (recombinantADENOVIRAL VECTOR))
S14		USPT	in vivo gene therapy and (bax pro-apoptotic gene and (recombinantADENOVIRAL VECTOR))
S13		USPT	bax pro-apoptotic gene and (recombinantADENOVIRAL VECTOR)
S12		USPT	bax pro-apoptotic gene
S11		USPT	pro-apoptotic genes
S10		USPT	recombinantADENOVIRAL VECTOR
S9		USPT	ADENOVIRAL VECTOR

Set	Items	Description
S1	359	BAX (S) ADENOVIR?
S2	133	S1 NOT PY>1998
S3	41	RD (unique items)
S4	6	S3 AND (BAX (3N) ADENOVIR?)
S5	158	BAX (4N) (RETROVIR? OR PLASMID? OR VECTOR?)
S6	65	S5 NOT PY>1998
S7	24	RD (unique items)
S8	150	BAX (S) REVIEW?
S9	67	S8 NOT PY>1998
S10	23	RD (unique items)

30,36
27,28,47

Abstract

Title: Gene therapy for hepatocellular carcinoma using two recombinant adenovirus vectors with alpha-fetoprotein promoter and Cre/ lox P system (ABSTRACT AVAILABLE)

Author(s): Sakai Y; Kaneko S (REPRINT) ; Sato Y; Kanegae Y; Tamaoki T; Saito I; Kobayashi K

Corporate Source: Kanazawa Univ,Sch Med, Dept Internal Med 1,13-1 Takara Machi/Kanazawa/Ishikawa 9208641/Japan/ (REPRINT); Kanazawa Univ,Sch Med , Dept Internal Med 1,Kanazawa/Ishikawa 9208641/Japan/; Univ Tokyo,Inst Med Sci, Mol Genet Lab, Minato Ku,Tokyo 1088639//Japan/; Univ Calgary,Dept Biochem & Mol Biol,Calgary/AB T2N 4N1/Canada/

Journal: JOURNAL OF VIROLOGICAL METHODS, 2001, V92, N1 (MAR), P5-17

ISSN: 0166-0934 Publication date: 20010300

Publisher: ELSEVIER SCIENCE BV, PO BOX 211, 1000 AE AMSTERDAM, NETHERLANDS

Language: English Document Type: ARTICLE

Abstract: Tissue-specific promoter has been used for cancer-specific suicide gene therapy, but its transcriptional activity is relatively low. For more efficient gene therapy of hepatocellular carcinoma, a simultaneous infection method of two recombinant adenoviruses was developed, in which one carried Cre gene under the control of a-fetoprotein promoter and the other a potent expression unit activated by Cre. When the vectors with lacZ reporter gene were introduced systematically into mouse models of disseminated tumors, specific and enhanced gene expression was observed exclusively in hepatocellular carcinomas both in the liver and in the lung. Next, using herpes simplex virus thymidine kinase, the anti-tumor effect was examined. Although in cultured cells, 60-300-fold expression of enzymatic activity and enhanced ganciclovir sensitivity was obtained compared with that of the single recombinant adenovirus directly driven by a-fetoprotein promoter, there was no significant anti-tumor effect for subcutaneous tumor on athymic mice. The lack of anti-tumor effect in mice could be explained by insufficient simultaneous transduction of the two vectors in the tumors, since it was found that a high multiplicity of infection was required to activate this system. Some strategies to overcome this dose limitation are needed, at least in the case of hepatocellular carcinoma.

Medicine, St. Louis, Missouri 63110, USA.
Journal of neurosurgery (UNITED STATES) Jan 1998, 88 (1) p99-105, ISSN 0022-3085 Journal Code: JD3
Contract/Grant No.: NS29477, NS, NINDS
Languages: ENGLISH
Document type: JOURNAL ARTICLE

OBJECT: Genes known to be involved in the regulation of apoptosis include members of the bcl-2 gene family, such as inhibitors of apoptosis (bcl-2 and bcl-xl) and promoters of apoptosis (bax). The authors investigated a potential approach for the treatment of malignant gliomas by using a gene transfection technique to manipulate the level of an intracellular protein involved in the control of apoptosis. METHODS: The authors transfected the murine bax gene, which had been cloned into a mammalian expression vector, into the C6 rat glioma cell line. Overexpression of the bax gene resulted in a decreased growth rate (average doubling time of 32.96 hours compared with 22.49 hours for untransfected C6, and 23.11 hours for clones transfected with pcDNA3 only), which may be caused, in part, by an increased rate of spontaneous apoptosis (0.77 +/- 0.15% compared with 0.42 +/- 0.08% for the vector-only transfected C6 cell line; p = 0.038, two-tailed Student's t-test). Treatment with 1 microM cytosine arabinoside (ara-C) resulted in significantly more cells undergoing apoptosis in the cell line overexpressing **bax** than in the **vector** -only control cell line (23.57 +/- 2.6% compared with 5.3 +/- 0.7% terminal deoxynucleotidyl transferase-mediated biotinylated-deoxyuridine triphosphate nick-end labeling technique-positive cells; p = 0.007). Furthermore, measurements of growth curves obtained immediately after treatment with 0.5 microM ara-C demonstrated a prolonged growth arrest of at least 6 days in the cell line overexpressing bax. CONCLUSIONS: These results can be used collectively to argue that overexpression of bax results in increased sensitivity of C6 cells to ara-C and that increasing bax expression may be a useful strategy, in general, for increasing the sensitivity of gliomas to antineoplastic treatments.

Apoptosis: pathophysiology of programmed cell death.
Bosman FT; Visser BC; van Oeveren J
Department of Pathology, Erasmus University Rotterdam, The Netherlands.
Pathology, research and practice (GERMANY) Jul 1996, 192 (7) p676-83, ISSN 0344-0338 Journal Code: PBZ
Languages: ENGLISH
Document type: JOURNAL ARTICLE; REVIEW; REVI **Cell death and cancer: replacement of apopt death suppressor genes in therapy.**
Favrot M; Coll JL; Louis N; Negoescu A
Lung Cancer Research Group, Institut Albert Bonniot, Faculte de Medecine, Universite Joseph Fourier, Grenoble, France.
Gene therapy (ENGLAND) Jun 1998, 5 (6) p728-39, ISSN 0969-7128 Journal Code: CCE
Languages: ENGLISH

The E1B 19K protein blocks apoptosis by interacting with and inhibiting the p53-inducible and death-promoting Bax protein.

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Center for Advanced Biotechnology and Medicine, Rutgers University, Piscataway, New Jersey 08854 USA.

Genes & development (UNITED STATES) Feb 15 1996, 10 (4) p461-77, ISSN 0890-9369 Journal Code: FN3

Contract/Grant No.: CA53370, CA, NCI; CA60088, CA, NCI

Languages: ENGLISH

Document type: JOURNAL ARTICLE

The E1B 19K protein is a potent apoptosis inhibitor and the putative **adenovirus** Bcl-2 homolog. To investigate the mechanism of apoptosis regulation, 19K-interacting cellular proteins were identified using the yeast two-hybrid system, and **Bax** was one of seven 19-K interacting clones. Residues 50-78 of **Bax** containing a conserved region designated Bcl-2 homology region 3 (BH3) were sufficient for specific binding to both the E1B 19K and Bcl-2 proteins. The **Bax** -E1B 19K interaction was detectable in vitro and in lysates from mammalian cells, and **Bax** expression antagonized E1B 19K protein function. **bax** mRNA and protein levels were p53-inducible with kinetics identical to that of p21/Waf-1/Cip-1, and E1B 19K and Bcl-2 expression did not affect **Bax** or p21/Waf-1/Cip-1 accumulation. In cells where p53 was mutant, **Bax** expression induced apoptosis, suggesting that **Bax** was sufficient for apoptosis, and acted downstream of p53. p53 may simultaneously activate the transcription of genes required for both growth arrest (p21/Waf-1/Cip-1) and death (**bax**), and E1B 19K and Bcl-2 may act distally and function through interaction with and antagonism of **Bax** to prevent apoptosis. With the death pathway disabled, induction of growth arrest by p53 can then be manifested.

Overexpression of Bax gene sensitizes K562 erythroleukemia cells to apoptosis induced by selective chemotherapeutic agents.

Kobayashi T; Ruan S; Clodi K; Kliche KO; Shiku H; Andreeff M; Zhang W

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Oncogene (ENGLAND) Mar 26 1998, 16 (12) p1587-91, ISSN 0950-9232 Journal Code: ONC

Contract/Grant No.: CA55164, CA, NCI; CA57639, CA, NCI; CA16672, CA, NCI

Languages: ENGLISH

Document type: JOURNAL ARTICLE

Bax and Bcl-2 are a pair of important genes that control programmed cell death, or apoptosis, with Bax being the apoptosis promoter and Bcl-2 the apoptosis protector. Although the detailed mechanism is unknown, the protein products of these two genes form protein dimers with each other and the relative ratio of the two proteins is believed to be a determinant of the balance between life and death. In our preliminary study, we found that K562 erythroleukemia cells have an extremely low level of endogenous Bcl-2 expression and a fairly high level of endogenous Bax expression. We constructed **Bax** and Bcl-2 expression **vectors** and transfected them into K562 cells. We found that transfection of **Bax vector** increased the expression of **Bax** protein; a shortened form of Bax also appeared. Cell death analysis using the Annexin V assay showed that the **Bax vector** caused significantly more apoptotic cells that the Bcl-2 or pCI-neo vector did. After selection with G418, Bax, Bcl-2 and pCI-neo stably transfected cells were established. These three cell lines were examined for their response to the chemotherapeutic agents ara-C, doxorubicin, etoposide and SN-38. Bax-K562 cells showed significantly higher fractions of apoptotic cells than pCI-neo-K562 cells when treated with ara-C, doxorubicin or SN-38. No sensitization effect was seen when etoposide was used. In contrast, Bcl-2-K562 cells had fewer apoptotic cells than pCI-neo-K562 cells after treatment with all these agents. Therefore, Bax may sensitize K562 cells to apoptosis induced by a wide range of, but not all, chemotherapeutic agents.

Transfection of C6 glioma cells with the bax gene and increased sensitivity to treatment with cytosine arabinoside.

Vogelbaum MA; Tong JX; Higashikubo R; Gutmann DH; Rich KM

Department of Neurological Surgery, Washington University School of

The E1B 19K protein blocks apoptosis by interacting with and inhibiting the p53-inducible and death-promoting Bax protein

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The E1B 19K protein is a potent apoptosis inhibitor and the putative adenovirus Bcl-2 homolog. To investigate the mechanism of apoptosis regulation, 19K-interacting cellular proteins were identified using the yeast two-hybrid system, and Bax was one of seven 19K-interacting clones. Residues 50–78 of Bax containing a conserved region designated Bcl-2 homology region 3 (BH3) were sufficient for specific binding to both the E1B 19K and Bcl-2 proteins. The Bax–E1B 19K interaction was detectable in vitro and in lysates from mammalian cells, and Bax expression antagonized E1B 19K protein function. *bax* mRNA and protein levels were p53-inducible with kinetics identical to that of *p21/Waf-1/Cip-1*, and E1B 19K and Bcl-2 expression did not affect Bax or p21/Waf-1/Cip-1 accumulation. In cells where p53 was mutant, Bax expression induced apoptosis, suggesting that Bax was sufficient for apoptosis, and acted downstream of p53. p53 may simultaneously activate the transcription of genes required for both growth arrest (*p21/Waf-1/Cip-1*) and death (*bax*), and E1B 19K and Bcl-2 may act distally and function through interaction with and antagonism of Bax to prevent apoptosis. With the death pathway disabled, induction of growth arrest by p53 can then be manifested.

[*Key Words*: Apoptosis; Bcl-2; E1B19K; p53; Bax; cell death]

Received October 12, 1995; accepted in revised form December 18, 1995.

Expression of the adenovirus[1] E1A gene stimulates host cell DNA synthesis, which creates a suitable environment for viral DNA replication (Moran 1993). The induction of DNA synthesis by E1A, however, cosegregates with the induction of apoptosis (White et al. 1991; Mymryk et al. 1994; Teodoro et al. 1995), which is responsible for impairing virus production (White 1994, 1995). The ability of E1A to induce apoptosis in rodent cells is p53-dependent. E1A expression causes p53 accumulation by increasing the half-life of p53 protein (Lowe and Ruley 1993; Chiou et al. 1994b), E1A transforms p53 −/− but not p53 +/+ mouse embryo fibroblasts (Lowe et al. 1994), and dominant-interfering mutant forms of p53 block the induction of apoptosis by E1A (Debbas and White 1993). Furthermore, BRK cells transformed by E1A and the temperature-sensitive p53(val135) [tsp53-(val135)] proliferate at the restrictive temperature when p53 is predominantly in the mutant conformation and undergo apoptosis at the permissive temperature when p53 is predominantly wild type (Debbas and White 1993; Chiou et al. 1994a; Lin et al. 1995; Sabbatini et al. 1995b). Thus, functional p53 is required for E1A-induced apoptosis.

[3]Corresponding author.

p53 is the most commonly mutated gene in human tumors (Hollstein et al. 1991; Vogelstein 1990) and encodes a transcription factor capable of both repression and *trans*-activation (Prives 1994). It is clear that p53 has a role in the induction of growth arrest through the *trans*-activation of the *p21/Waf-1/Cip-1* gene, the product of which inhibits cell cycle-dependent kinases (El-Deiry et al. 1993; Harper et al. 1993; Xiong et al. 1993; Noda et al. 1994) which may contribute to the tumor suppression activity of p53.

Another mechanism by which p53 could act as a tumor suppressor is realized with the observation that in some circumstances p53 does not induce growth arrest but, rather, cell death through apoptosis (Yonish-Rouach et al. 1991). Thus, p53 can function as a tumor suppressor by inducing either growth arrest or cell death, depending on the physiological circumstances. p53 may be a cellular surveillance factor that responds to DNA damage or the deregulation of cell growth control, as is the case with E1A. p53 may thereby play an important role in triggering cell death as a means of eliminating virus-infected or transformed cells.

How p53 implements the apoptotic program is still not clear and may differ with varying physiological circumstances. In BRK cells transformed by E1A and the

tsp53(val135), the activity of p53 as a transcription factor is required for apoptosis. Inactivating mutations in the transcriptional activation domain of p53 prevent induction of apoptosis when p53 assumes the wild-type conformation (Sabbatini et al. 1995b). In this setting p53 may activate the transcription of death genes or repress the transcription of survival genes to promote apoptosis.

To prevent the detrimental E1A-induced, p53-dependent apoptosis, the adenovirus-encoded E1B gene produces two different gene products, E1B 55K and E1B 19K, to disable p53 function. Although it is clear that E1B 55K blocks the function of p53 by complexing with and inhibiting transcriptional activity of p53 (Sarnow et al. 1982; Yew and Berk 1992), the mechanism by which E1B 19K inhibits p53-mediated apoptosis has not been determined. However, the E1B 19K protein does share functional and structural homology with Bcl-2, suggesting that the two proteins may act by similar mechanisms to inhibit apoptosis (Rao et al. 1992; Boyd et al. 1994; Chiou et al. 1994b).

The E1B 19K and Bcl-2 proteins are functionally interchangeable in a variety of different settings. Bcl-2, for example, will substitute for the E1B 19K protein and will cooperate with E1A to transform rodent cells (Rao et al. 1992). Both the E1B 19K and Bcl-2 proteins block apoptosis induced by p53 (Chiou et al. 1994a; Lin et al. 1995; Sabbatini et al. 1995a), and Bcl-2 will functionally substitute for the E1B 19K protein during adenovirus infection of human cells (Tarodi et al. 1993; Chiou et al. 1994b). However, the E1B 19K protein is more effective at inhibiting apoptosis mediated by tumor necrosis factor α (TNF-α) and Fas antigen (Gooding et al. 1991; Hashimoto et al. 1991; White et al. 1992). Thus, the E1B 19K protein is an apoptosis inhibitor and is functionally very similar to Bcl-2, suggesting that the 19K protein may be a member of the Bcl-2 family of apoptosis regulators (Chiou et al. 1994b).

The E1B 19K protein shares limited sequence homology with other members of the Bcl-2 family. The highly conserved central region of the E1B 19K protein has been defined by mutational analysis to be important in transformation and regulation of apoptosis (White et al. 1992; Chiou et al. 1994b). A comparison of the amino acid sequence of Bcl-2 and E1B 19K proteins has indicated amino acid sequence homology in the region of 19K known to be important for structure and function, particularly within Bcl-2 homology region 1 (BH1) (White et al. 1992; Chiou et al. 1994b).

The E1B 19K and Bcl-2 proteins also share the ability to block apoptosis induced by p53. The introduction of an E1B 19K or Bcl-2 expression vector into E1A plus tsp53(val135)-transformed BRK cell lines completely prevents the induction of apoptosis by p53 at the permissive temperature and causes cells to remain in a growth-arrested state (Debbas and White 1993; Chiou et al. 1994a; Lin et al. 1995; Sabbatini et al. 1995a). There is a complete cessation of DNA synthesis with growth arrest occurring primarily in the G_2 phase of the cell cycle (Chiou et al. 1994a; Lin et al. 1995). Thus, E1B 19K and Bcl-2 proteins are functionally interchangeable for blocking p53-mediated apoptosis, but the p53-dependent growth arrest function remains intact, suggesting that the apoptosis and growth arrest functions of p53 are separable.

Because the biochemical mechanism of E1B 19K protein function was unknown, we screened a HeLa cell cDNA library using the yeast two-hybrid system for E1B 19K-interacting proteins. We demonstrate here that one of the E1B 19K binding proteins is Bax. Bax was identified previously as a Bcl-2-interacting protein that prevents inhibition of apoptosis by Bcl-2 (Oltvai et al. 1993). Bax similarly antagonizes the ability of the 19K protein to inhibit apoptosis. Furthermore, *bax* is p53-inducible and sufficient for activating the apoptosis pathway in the absence of functional p53. p53 may act as a tumor suppressor by inducing the transcription of genes required for both apoptosis and growth arrest. Bcl-2 and E1B 19K proteins may function by inhibiting apoptosis through specific Bax interaction and thereby control the physiological outcome of p53 function.

Results

Isolation of Bax from a two-hybrid screen for E1B 19K-interacting proteins

To identify E1B 19K-binding proteins that may play a critical role in the regulation of apoptosis, we screened a HeLa cell cDNA library constructed in the pGAD–GH vector, which has cDNA library sequences fused to the GAL4 activation domain. The bait plasmid consisted of the *E1B 19K* open reading frame fused to the sequence for the yeast GAL4 DNA-binding domain in the pGBT-9 vector. Three million transformants were screened for growth in the absence of histidine and production of β-galactosidase by an X-gal filter assay. Seven positive clones (Bp1–7) that interacted strongly with the E1B 19K protein did not show the interaction when combined with the pGBT-9 vector alone or an irrelevant gene (*apc-2*) in pGBT-9, indicating specificity for interaction with the E1B 19K protein (Fig. 1). Sequence analysis of the clones revealed that Bp3 was identical in sequence with part of exon 3 of human *bax* (*hbax*) encoding amino acids 50–78 in frame with the activation domain of GAL4 (see Fig. 1C). The exon 3 sequences in Bp3 were followed by a novel open reading frame encoding 30 additional amino acids (designated exon 4′) prior to a stop codon. *bax* exon 4 sequences were followed out of frame before reaching an untranslated region and polyadenylation sequence (Fig. 1C).

The strong interaction of Bp3 with E1B 19K in the two-hybrid system suggested that residues 50–78 of *bax* exon 3 may encode the E1B 19K-binding site. For this reason, two pGAD–GH constructs were generated: one that encoded residues 50–78 of *bax* exon 3 with the novel exon 4′ sequences but without the untranslated region including exon 4 (Bp3–Ex3/4′); and the second encoded only residues 50–78 of *bax* exon 3 (Bp3–Ex3). Both constructs showed strong interactions with the 19K protein (Fig. 1A for Bp3–Ex3; data not shown for Bp3–

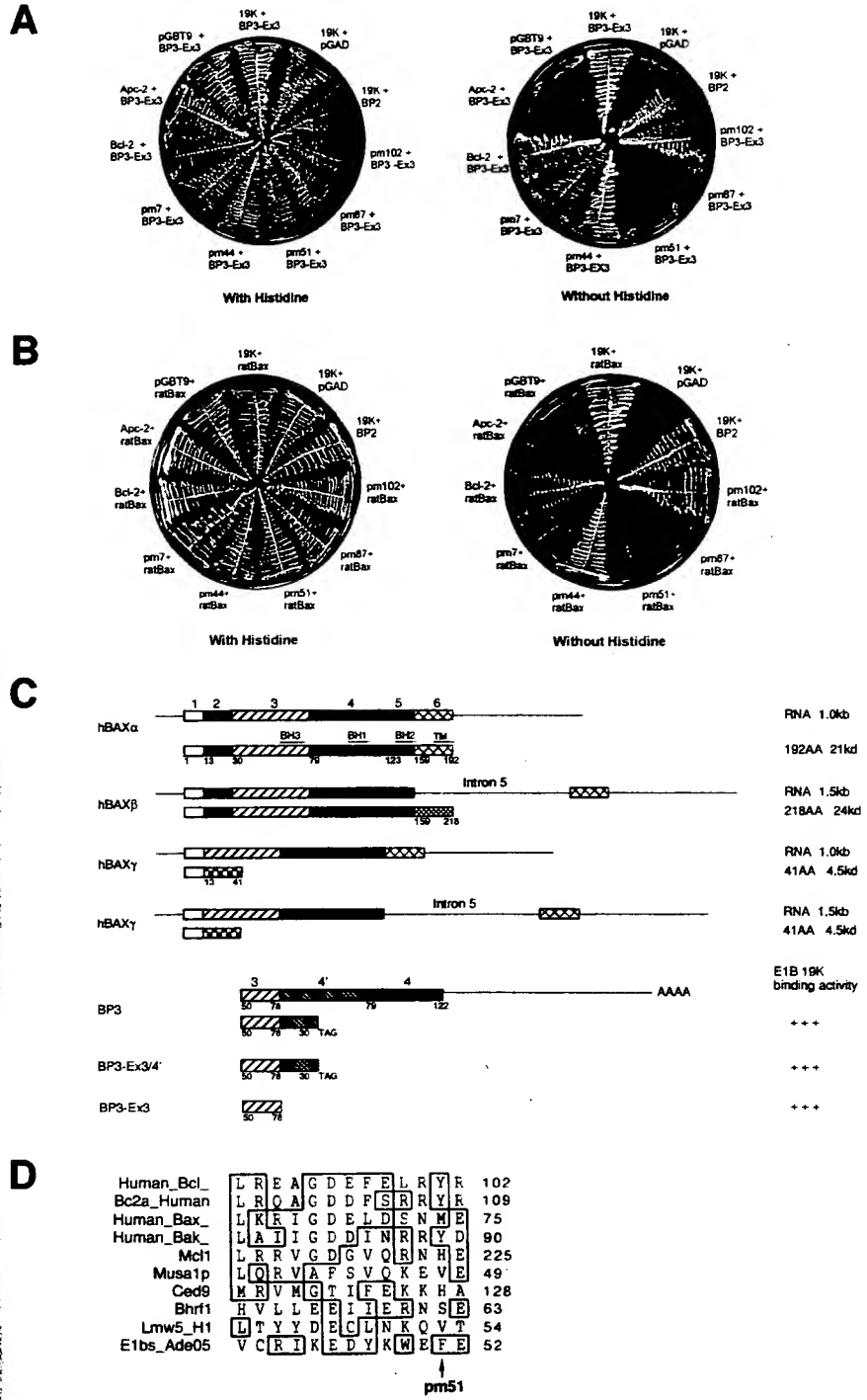


Figure 1. Interaction of the E1B 19K protein with Bax in yeast. (*A*) Two-hybrid interaction assay in yeast strain YGH1 with plasmids expressing the GAL4 activating domain–Bp3–Ex3 (amino acid 50–78 of hBax) fusion protein in combination with plasmids expressing a GAL4 DNA-binding domain (pGBT-9) fused to either the E1B 19K protein (19K), an irrelevant protein (Apc-2), the full-length Bcl-2 protein (Bcl-2), or the E1B 19K missense mutants pm7, pm44, pm51 (BH3 mutant), pm87 (BH1 mutant), and pm102. Bp2 is located 252 amino acids to the carboxyl terminus of lamin A fused to the activation domain of GAL4, which was also isolated from the two-hybrid screen based on the ability to interact with the E1B 19K protein. pGAD–GH is the control vector expressing the GAL4 activation domain alone. The + histidine plate lacks only leucine and tryptophan, thus selecting only for the presence of pGAD–GH and pGBT-9. The − histidine plate lacks leucine, tryptopan, and histidine, thus selecting not only for the presence of the two plasmids but also for the interaction of the plasmid-encoded fusion proteins. (*B*) Two-hybrid interaction assay in yeast strain YGH1 with plasmids expressing the GAL4 activating domain–rBax fusion protein in combination with the plamids described above. (*C*) Schematic representation of alternative transcripts of *bax* and their predicted protein products. Exons are indicated by boxes. Because of the alternative splicing of exon 2 in Baxγ, exon 3 in Baxγ is different from exon 3 in Baxα, as indicated by the different shading pattern. The BH3, BH1, and BH2 conserved regions of the Bcl-2 family are indicated. (TM) The transmembrane region. (BP3) The Bax segment isolated from the two-hybrid screen for 19K association. Bp3–Ex3/4′ and Bp3–Ex3 constructs were derived from BP3 as indicated. (+ + +) Interaction of Bax constructs with the E1B 19K protein in yeast as in *A* and *B*. (*D*) Amino acid sequence alignment of the BH3 region among members of the Bcl-2 family.

Ex3/4′). Moreover, all three constructs (Bp3, Bp3–Ex3/4′, Bp3–Ex3) displayed equally strong interactions with Bcl-2 in yeast (Fig. 1A for Bp3–Ex3; data not shown for Bp3-Ex3/4′ and Bp3), suggesting that the binding requirements for both 19K and Bcl-2 were similar, if not identical, and Bp3–Ex3 was sufficient for the interaction to occur in yeast.

Because Bp3, Bp3–Ex3, and Bp3–Ex3/4′ all encode segments of Bax, interaction of the E1B 19K protein with full-length Bax was evaluated. Full-length rat *bax* α *(rbax)* was cloned by reverse transcription and PCR of mRNA from the BRK cell line p53An1 (Debbas and White 1993) and was tested for interaction with the E1B 19K protein in yeast (Fig. 1B). rBax possessed the same capacity to interact with the 19K protein as Bp3–Ex3 and other Bax constructs (Fig. 1B). As expected, full-length

rBax also interacted with Bcl-2 (Fig. 1B). Thus, rBax interacted with E1B 19K and residues 50–78 of Bax were sufficient for interaction not only with the E1B 19K protein but also the Bcl-2 protein in yeast.

Sequence requirements within the E1B 19K protein for Bax interaction

The interaction of Bax with specific E1B 19K mutants known to be defective for E1B 19K function and/or structure was used as a measure of the specificity of the interaction. We tested the ability of the four constructs (Bp3, Bp3–Ex3, Bp3–Ex3/4′, and rBax in pGAD) to interact with missense mutants of the E1B 19K protein (pm7, pm44, pm51, pm87, and pm102) which resulted in a loss of transforming activity and the ability to block apoptosis induced by TNF-α and Fas antigen (White et al. 1992; Chiou et al. 1994b).

Substitution of either phenylalanine for serine at position 51 (pm51) or glycine for alanine at position 87 (pm87) in the E1B 19K protein resulted in loss of the ability to interact with Bp3, Bp3–Ex3, Bp3–Ex3/4′, and rBax in pGAD in yeast (Fig. 1). pm7, pm44, and pm102, however, maintained the ability to interact with the Bax constructs, except that pm7 interacted more weakly with rBax (Fig. 1). pm87 has a replacement of a highly conserved glycine (100% identical across all 12 adenovirus serotypes) with alanine in the region of the 19K protein homologous to BH1 (White et al. 1992; Chiou et al. 1994b). The same glycine to alanine substitution in BH1 of Bcl-2 prevents both Bcl-2 function and Bcl-2–Bax interaction (Yin et al. 1994). Thus, the equivalent BH1 mutation in the E1B 19K protein (pm87) also prevents Bax interaction. pm51 is located within another highly conserved region of the E1B 19K protein and other members of the Bcl-2 family that we have designated Bcl-2 homology region 3 (BH3) (Fig. 1C and D). The BH3 region is also included in residues 50–78 of Bax, which is sufficient for the 19K protein interaction in yeast. Thus, sequence information within BH3 of Bax is necessary and sufficient for interaction with the E1B 19K and Bcl-2 proteins. Likewise, sequence information within BH3 of the E1B 19K protein is apparently also required for interaction with Bax.

Having established that point mutations in pm51 and pm87 result in a loss of interaction between E1B 19K and Bax (Fig. 1), we made further deletions in the E1B 19K protein to map the minimal region sufficient for the interaction. Sequential deletions from both the amino-terminal (ΔN30, ΔN64, and ΔN87) and carboxy-terminal (ΔC146, ΔC93, ΔC70) ends were generated in addition to four mutants with deletions of both amino- and carboxy-terminal ends (30–146, 30–93, 64–146, 19–57) (Fig. 2). Both full-length rBax and Bp3–Ex3 were tested with the E1B 19K deletion mutants in the two-hybrid system. Interactions were confirmed by the ability of yeast to grow in the absence of histidine and turn blue in a β-galactosidase assay.

Deletions of either the amino-terminal 64 residues (ΔN64) or the carboxy-terminal 30 amino acids (ΔC146) of the E1B 19K protein still permitted interaction with Bp3–Ex3 as effectively as the wild-type E1B 19K protein (Fig. 2). Correspondingly, the central domain containing deletion 30–146 was sufficient for the interaction whereas larger deletions of either the amino or carboxyl terminus of the E1B 19K protein were not capable of interacting with Bp3–Ex3 (Fig. 2). Thus, the minimal region of the E1B 19K protein required for binding to Bp3–Ex3 maps to the central conserved domain, including BH1, BH2, and BH3 (Fig. 2). Interestingly, the 19–57 fragment of the E1B 19K protein that corresponds to Bp3–Ex3 did not interact with Bp3–Ex3 (Fig. 2). The inability of equivalent regions with the 19K and Bax proteins to interact suggests that protein–protein interactions may be nonsymmetrical.

Interaction of the E1B 19K protein with full-length rBax produced somewhat different results. None of the E1B 19K deletions that interacted with Bp3–Ex3 (ΔN64, ΔC146, or 30–146) interacted with full-length rBax (Fig. 2). The 19K deletion 19–57 containing BH3, however, was sufficient for interacting with full-length rBax, albeit weakly (Fig. 2). None of the other deletions, all of which lack the BH3 region, showed any interaction. Taken together, these results suggest that the BH3 region is required but not sufficient for establishing the interaction between E1B 19K and Bax proteins.

E1B 19K associates with Bax in vitro and in cell lysates

In parallel with the yeast two-hybrid assay for 19K–Bax interaction, we examined the ability of the E1B 19K and Bax proteins to interact in vitro. hBcl-2, E1B 19K, and rBax were prepared by in vitro transcription and cotranslation and assayed for the ability to coimmunoprecipitate. Following cotranslation, the ^{35}S-labeled hBcl-2, E1B 19K, and rBax proteins were incubated in specific combination with antibodies directed against Bcl-2 (C21), Bax (N-20), and E1B 19K (p21) proteins. The Bcl-2 antibody precipitated in vitro-synthesized Bcl-2 protein but not the E1B 19K and Bax proteins (Fig. 3A). When the E1B 19K and Bcl-2 proteins were cotranslated, the Bcl-2 antibody did not pull out the E1B 19K protein, indicating that there is no interaction between the Bcl-2 and 19K proteins. This result was expected, as there is no detectable interaction between the E1B 19K and Bcl-2 proteins when assayed in the yeast two-hybrid system (data not shown). However, when Bcl-2 and Bax were cotranslated, immunoprecipitation with a Bcl-2-specific antibody coprecipitated Bax (Fig. 3A). Bax migrated with a slightly higher molecular mass (22.5 kD rather than 21 kD) because of the Myc epitope tag. Immunoprecipitation with a polyclonal antibody specific for the E1B 19K protein specifically precipitated the E1B 19K protein and not Bax or Bcl-2 (Fig. 3A). The E1B 19K and Bcl-2 proteins also did not coimmunoprecipitate with the E1B 19K specific antibody, as was the case with the Bcl-2-specific antibody (Fig. 3A). When the E1B 19K and Bax proteins were cotranslated, however, both E1B 19K and Bax were coprecipitated (Fig. 3A). In the converse experiment, [illegible]

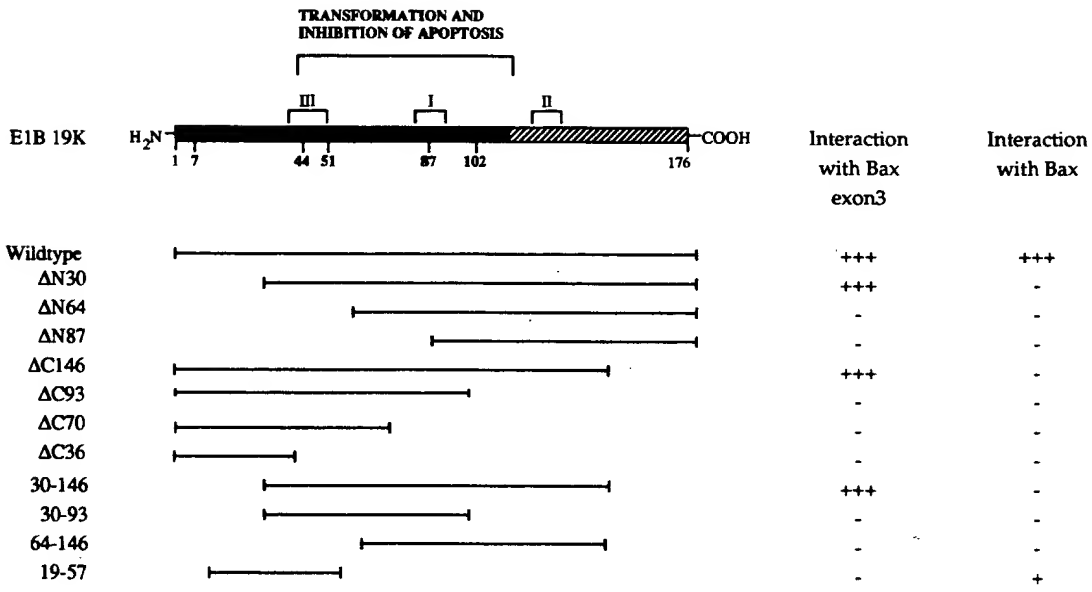


Figure 2. Mapping of the minimal region of the E1B 19K protein required for interaction with Bp3–Ex3 (amino acids 50–78 of hBax) and rBax. A schematic representation of the 176 amino acids of wild-type E1B 19K protein highlights some of its structural features. Among the various adenovirus serotypes, amino acids 1–81 (stippled area) share 52% identity, amino acids 82–113 (solid area) is the most conserved region with 63% identity and amino acids 114–176 (hatched area) is least conserved with 37% identity (Chiou et al. 1994b). The region between amino acids 44 and 113 has been determined previously as being required for transformation and inhibition of apoptosis and is indicated (White et al. 1992; Chiou et al. 1994b). Boxed regions I, II, and III define the Bcl-2 homology regions BH1, BH2, and BH3, respectively. Locations of the point mutants of E1B 19K tested in the two-hybrid system (pm7, pm44, pm51, pm87, and pm102) are in boldface type. Deletion mutants of the E1B 19K protein were generated from the amino-terminal direction (ΔN30, ΔN64, ΔN87), the carboxy-terminal direction (ΔC146, ΔC93, ΔC70, ΔC36), and both directions (30–146, 30–93, 64–146, 19–57) and tested for interaction with either full-length rBax or Bp3-Ex3 in the two-hybrid system. Positive interactions were scored by the number of colonies obtained on plates without in comparison to plates containing histidine and by the intensity of blue color in a β-galactosidase assay. (+ + +) Strong interaction; (+) weak interaction; (−) no interaction.

ation of E1B 19K and Bax, followed by immunoprecipitation with an anti-Bax antibody, also coimmunoprecipitated E1B 19K with Bax (Fig. 3A). Bax also coimmunoprecipitated with Bcl-2, as expected (Fig. 3A). These results indicate that the E1B 19K protein can specifically interact with Bax in vitro, which serves as an independent confirmation of results obtained from the yeast two-hybrid system.

To further substantiate the in vitro interactions observed, a glutathione *S*-transferase (GST) fusion protein system was employed. GST–19K, a GST fusion of E1B 19K GST–pm51, a GST fusion of a E1B 19K mutant within the BH3 region defective for Bax interaction in yeast, and GST alone were immobilized onto glutathione–Sepharose beads, purified, and used to confirm the interaction between the E1B 19K and Bax proteins (Fig. 3B). Equal amounts of GST–fusion proteins (3 μg) were incubated with either Bax or Bcl-2 proteins translated in vitro (Fig. 3C). In vitro-translated Bax bound to the GST–19K fusion protein but did not bind to either GST alone or the E1B 19K point mutant GST–pm51 (Fig. 3C). However, in vitro-translated Bcl-2 did not bind to GST–19K, GST–pm51, or GST alone (Fig. 3C). Thus, Bax specifically interacted with the E1B 19K protein in this assay, and complex formation with Bax was abrogated by a single amino acid substitution in the region identical to that in the two-hybrid assays required for 19K/Bax interaction. To verify the protein band as Bax, a portion of the in vitro-translated protein mix was immunoprecipitated with a monoclonal antibody specific for the Myc epitope tag on rBax, as opposed to an E1B 55K antibody (13D2) (Fig. 3C). In vitro-translated Bcl-2 was also immunoprecipitated by using a Bcl-2 polyclonal antibody as opposed to a nonspecific antibody (Fig. 3C). In each case, in vitro-translated protein was immunoprecipitated with its corresponding antibody as opposed to a nonspecific antibody.

The association of the E1B 19K and Bax proteins in the yeast two-hybrid system and the ability of the E1B 19K and Bax proteins to complex in vitro suggested that they may interact in mammalian cells. Detection of protein–protein complexes has been successful previously through coimmunoprecipitation of cellular proteins from cell lysates prepared under mild lysis conditions. The E1B 19K protein, however, is insoluble when expressed in mammalian cells and generally requires harsh detergent conditions to be extracted from cells for immunoprecipitation (White et al. 1984; White and Cipriani 1990). These conditions, however, are usually not suitable for protein interactions to remain intact. Conditions used to coimmunoprecipitate Bax complexed to Bcl-2 (Oltvai et al. 1993), for example, immunoprecipi-

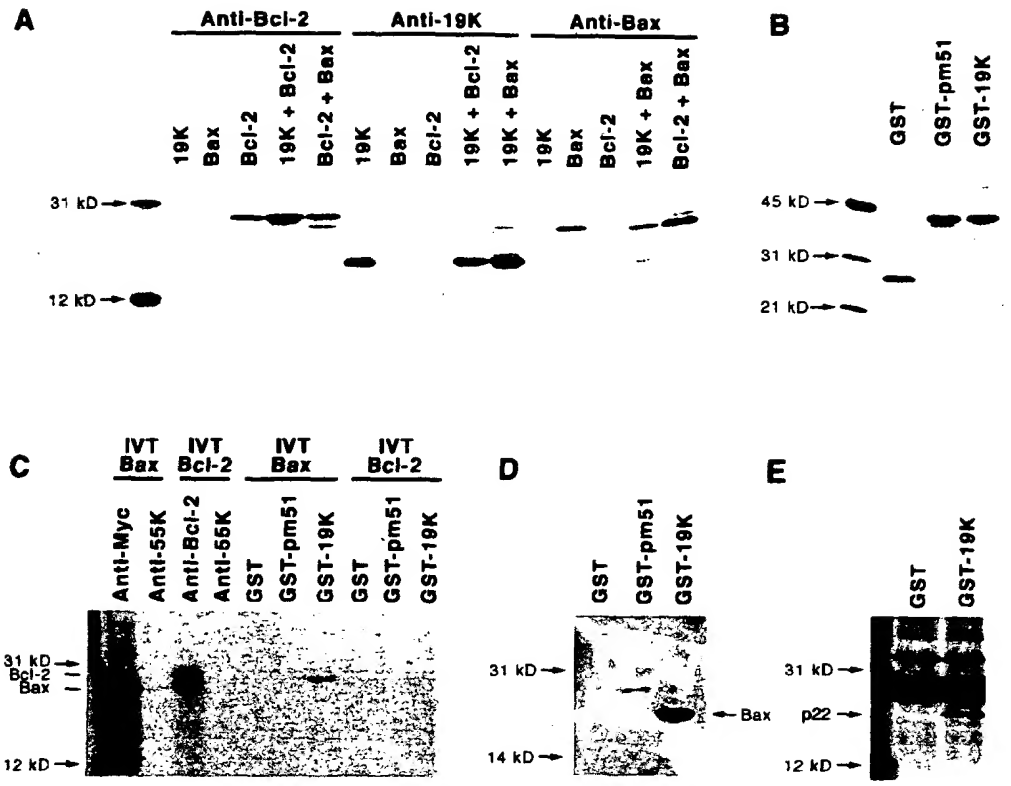


Figure 3. Association of the E1B 19K protein with rBax in vitro and in cell lysates. (*A*) Coimmunoprecipitation of in vitro-translated rBax and E1B 19K proteins. Immunoprecipitation was carried out with in vitro-synthesized and [^{35}S]methionine-labeled Bcl-2, E1B 19K, and Bax, in the specific combinations indicated, with antibodies directed against Bcl-2, Bax, and E1B 19K proteins. (*B*) Production of GST–fusion proteins. GST–19K, GST–pm51, and GST alone were produced in *Escherichia coli* and purified on glutathione–Sepharose beads, and the levels and purity of the GST fusions were evaluated by SDS-PAGE. Equal concentrations (3 μg) of GST–19K, GST–pm51, and GST were used for the binding experiments in *C* and *D*. (*C*) In vitro-translated rBax associates with the GST–19K fusion protein. GST–fusion proteins were immobilized onto glutathione–Sepharose beads and incubated with in vitro-translated rBax or hBcl-2 in the presence of NETN buffer as indicated. Immunoprecipitation of in vitro-translated rBax (Myc Ab) and hBcl-2 (Bcl-2 Ab) is shown. An antibody directed against the E1B 55K protein (13D2) was used as a control for nonspecific precipitation. (*D*) Interaction of the cellular rBax protein with the GST–19K fusion protein. Cold cell lysates prepared from the An1 cell line were incubated with GST–fusion proteins GST–19K and GST–pm51 immobilized on glutathione beads. Proteins were analyzed by SDS-PAGE and Western blotting with an anti-Bax polyclonal antibody. (*E*) Interaction of [^{35}S]methionine-labeled BRK cell proteins with GST and GST–19K. Labeled cell extracts of the An1 cell line were indubated with GST or GST–19K, and the bound products were alanized by SDS-PAGE. A protein with a molecular mass of ~22kD (p22) was the only GST–19K-specific band. p22 comigrated with Bax (*D*).

tate <1% of the 19K protein from cell lysates (data not shown). To circumvent this problem, the E1B 19K protein was immobilized onto glutathione–Sepharose beads as a GST–fusion protein (GST–19K) and then incubated with unlabeled lysates prepared from the E1A plus tsp53(val135)-transformed BRK cell line An1 (Debbas and White 1993), under conditions where Bax was expressed (see Western blot in Fig. 6, below). Cell lysates were incubated with GST alone, GST–pm51, or the GST–19K fusion proteins bound to beads in a GST pull-down assay. Bound cellular proteins were subjected to immunoblotting analysis by probing with an anti-Bax polyclonal antibody. rBax was specifically detected bound to the GST–19K fusion protein but not to either GST alone or the E1B 19K mutant GST–pm51 (Fig. 3D).

To further evaluate the specificity of the Bax interaction with GST–19K protein, a [^{35}S]methionine-labeled BRK cell extract was incubated with either GST or GST–19K, and the number of bound cellular proteins was examined. There was a single specific [^{35}S]methionine-labeled band migrating with an approximate molecular mass of 22kD (p22) that bound to GST–19K but not GST (Fig. 3E). p22 migrated in the expected position of Bax (see Fig. 3D), indicating that the interaction of cellular proteins with GST–19K was highly selective and that Bax interacts with high specificity. These results demonstrate that the E1B 19K protein can associate with Bax specifically in the context of a whole cell extract, suggesting that an in vivo interaction between the two proteins is probable and supports the findings obtained in the two-hybrid system and the in vitro coassociation of E1B 19K and Bax.

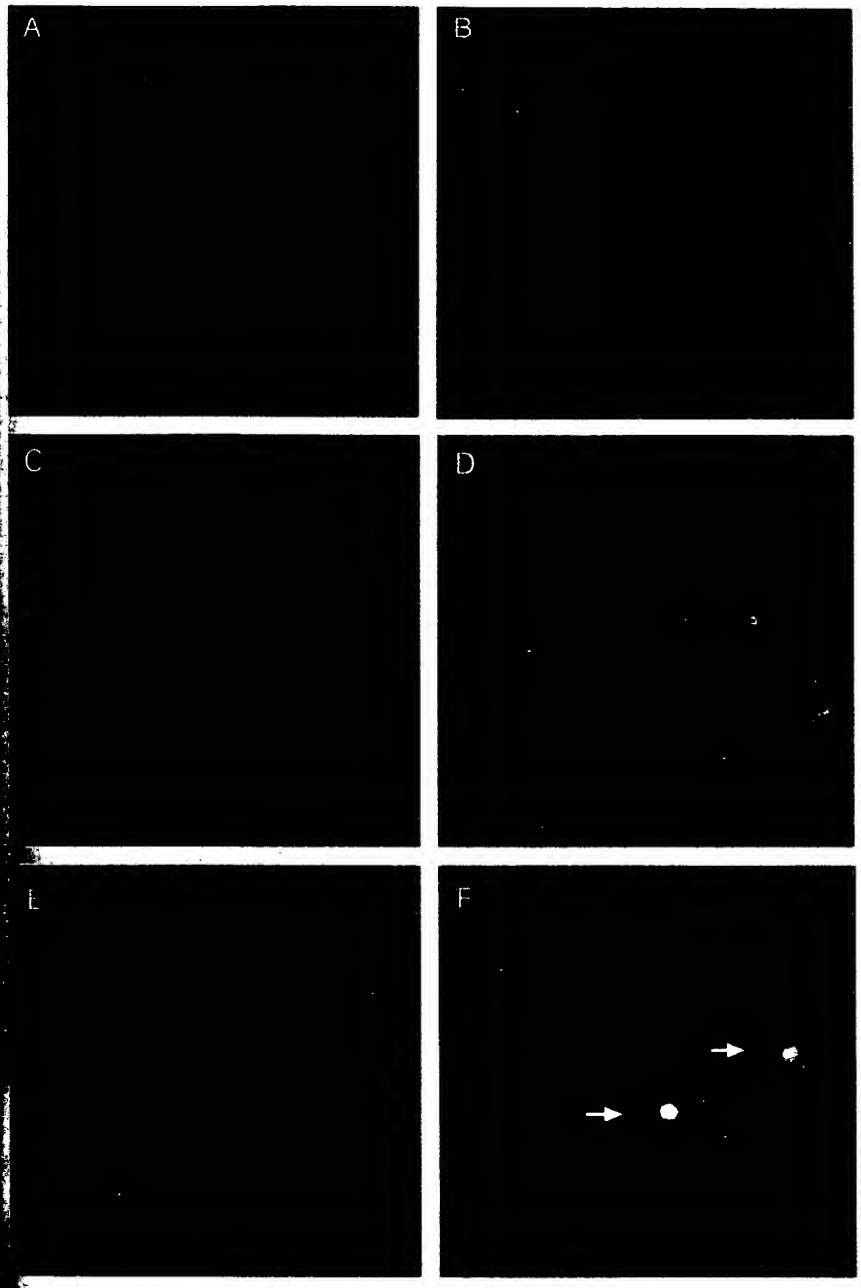


Figure 4. E1B 19K and Bax display overlapping intracellular localization. The 19K1 cell line was transfected with pCEP4–Myc–rBax expression vector and processed for double-label indirect immunofluorescence 48 hr post-transfection. The cells were stained with a monoclonal antibody directed against the Myc epitope tag on rBax (*A*, *C*) and a polyclonal antibody directed against the E1B 19K protein (*B*,*D*). (*E*,*F*) Induction of DNA fragmentation by transient Bax expression in BRK cells. The TdT assay to detect DNA fragmentation in situ was used in vector control (*E*) or CMV–Bax-transfected (*F*) 19K1 cells. Labeling with TdT is indicative of cells undergoing apoptotic cell death (arrows in *F*). Original magnification, 600×.

1B 19K and Bax proteins have overlapping ntracellular localization

he E1B 19K protein is found associated with the cytolasmic and nuclear membranes and with the insoluble uclear lamina (White et al. 1984; White and Cipriani 990). To determine whether the Bax and E1B 19K proeins reside in the same subcellular compartment, doule-label indirect immunofluorescence studies were perormed. Transient Bax expression induced apoptosis in RK cells that did not express the E1B 19K protein (data ot shown); therefore, the cytomegalovirus (CMV) Mycagged rBax expression vector (pCEP4–Myc–rBax) was lectroporated into the 19K1 cell line, which is an E1A plus tsp53(val135)-transformed BRK cell line that stably expresses the E1B 19K protein (Debbas and White 1993). Cell death following Bax expression was delayed sufficiently in the 19K1 cell line to permit visualization of Bax and 19K localization. The pCMV–rBax (pCEP4–Myc–rBax)-transfected 19K1 cells were fixed 48 hr post-transfection and stained with a monoclonal antibody specific for the Myc epitope on rBax and a polyclonal antibody directed against the E1B 19K protein. As reported previously (White et al. 1984; White and Cipriani 1989; White and Cipriani 1990), the E1B 19K protein was detected primarily at the nuclear envelope, and cytoplasmic membranes were typified by bright punctate cytoplasmic staining (Fig 4B and D). Half of the Bax positive

cells displayed colocalization of the two proteins typical of cytoplasmic membranes (Fig. 4A, B). The 19K protein is membrane and cytoskeletal bound (White et al. 1984), and Baxα possess a transmembrane domain and would be expected to be membrane bound also (Oltvai et al. 1993). The remaining cells had an overlapping staining pattern with the E1B 19K protein (Fig. 4C, D). These data suggested that a substantial fraction of Bax protein in vivo may reside in the same intracellular compartment as the E1B 19K protein.

Bax expression was eventually lethal in 19K1 cells, as evidenced by pronounced morphological changes and the loss of cell viability (see below) that accumulated with time following transfection and Bax protein expression. To address whether the cell death induced by transient Bax expression resembled apoptosis, a terminal deoxynucleotidyl transferase (TdT) assay (Gavrieli et al. 1992) was performed to detect the presence of fragmented DNA. Vector control-transfected cells were viable and had a low incidence of TdT-positive cells (Fig. 4E). The CMV–Bax-transfected cells, however, displayed a frequency of TdT-positive cells with condensed nuclei severalfold higher (Fig. 4F), suggesting that the cell death induced by Bax occurred by an apoptotic process.

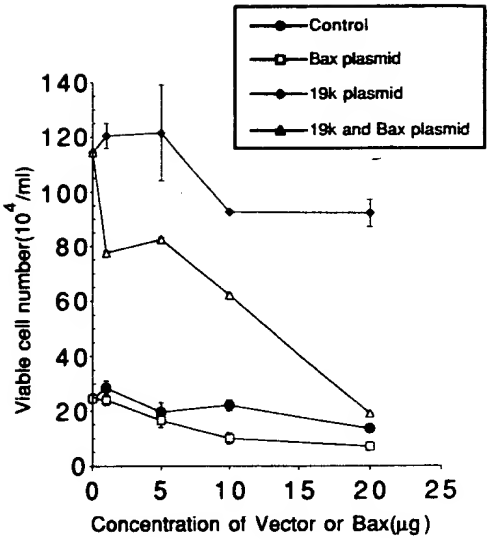


Figure 5. Bax antagonizes the antiapoptotic function of the E1B 19K protein. Increasing concentrations (1, 5, 10, and 20 μg) of the rBax expression vector DNA (pCEP4–Myc–rBax) or the control empty vector DNA were cotransfected into the An1 cell line with a fixed concentration (10 μg) of pcDNA3-19K DNA or the control vector DNA. The transfected An1 cell lines were incubated at 38.5°C for 60 hr and then at 32°C for 60 hr. Viable cell number was assessed by trypan blue exclusion.

Bax antagonizes apoptosis inhibition by the E1B 19K protein

Because rBax interacted with the E1B 19K protein in yeast and in vitro, we tested whether rBax could antagonize the antiapoptotic function of the E1B 19K protein in BRK cells. The E1A plus tsp53(val135)-transformed BRK cell line An1 is transformed at the restrictive temperature but undergoes apoptosis at the permissive temperature (Debbas and White 1993). Transient E1B 19K expression, however, protects the An1 cell line from p53-mediated apoptosis at the permissive temperature as measured by an increase in cell survival greater than fivefold at 60 hr post-transfection (Fig. 5). To determine whether Bax can reverse this E1B 19K protection against Bax-mediated apoptosis, the Bax expression vector pCEP4–Myc–rBax was transfected in increasing amounts with respect to a constant level of the E1B 19K expression vector pcDNA3–19K. The An1 cell line was also transfected with the rBax expression vector alone as a control. The transfected cells were incubated at the restrictive temperature to permit Bax and E1B 19K protein expression for 60 hr and were then shifted to the permissive temperature for 60 hr. The viable cell number was assessed by trypan blue exclusion.

In the absence of 19K expression, viability was reduced to 5% because of massive apoptosis at the permissive temperature (Fig. 5). Transfection of the rBax expression vector did not increase apoptosis measurably under these conditions of 95% cell death (Fig. 5). Cotransfection of increasing concentrations of the rBax expression vector with the E1B 19K expression vector, however, greatly diminished survival conferred by E1B 19K expression (Fig. 5). When the rBax vector DNA was transfected at a 2:1 ratio to 19K vector DNA, viability was reduced to the level of the control. Reduction of rBax vector levels relative to E1B 19K vector levels proportionally increased the level of protection from cell death by the E1B 19K protein (Fig. 5). Therefore, rBax protein antagonized the antiapoptotic function of the E1B 19K protein, suggesting an in vivo functional interaction between these two apoptosis regulators.

Wild-type p53 induces bax *mRNA and protein expression*

Recent evidence indicates that *bax* is a p53-inducible gene and that *bax* expression parallels p53-mediated apoptosis in certain cell types (Miyashita et al. 1994; Miyashita and Reed 1995). Therefore, we wanted to determine whether *bax* gene expression was up-regulated in BRK cell lines that succumb to p53-mediated apoptosis at the permissive temperature. We have demonstrated recently that E1A-induced apoptosis in BRK cells requires a transcriptionally functional p53 (Sabbatini et al. 1995b). The BRK cell line 22-23val135A, which expresses E1A and a transcriptionally defective tsp53 (22-23val135), was used as a negative control for p53-mediated *trans*-activation at the permissive temperature. tsp53(22-23val135) is transcriptionally defective because of a double mutation within the *trans*-activation domain of p53 (Lin et al. 1994; Sabbatini et al. 1995b). Accordingly, this cell line does not succumb to p53-mediated apoptosis at the permissive temperature (Sabbatini et al. 1995b). Cytoplasmic RNA was extracted at increasing

time points at the permissive temperature and then subjected to Northern blot analysis.

Results indicate that *rbax* mRNA levels increased three- to fivefold in the An1a and An1b cell lines [two E1A plus tsp53(val135)-transformed BRK cell lines] within 4 hr of incubation at the permissive temperature (Fig. 6A). *rbax* mRNA levels remained at induced levels in the An1a cell line after 9 hr of incubation at the permissive temperature but appeared to decrease in the An1b cell line at this time point (Fig. 6A). However, this sudden decrease in *rbax* mRNA levels in the An1b cell line is likely attributable to lower overall levels of mRNA because of cell death, as it was paralleled by a corresponding decrease in the levels of actin mRNA (Fig. 6A). Expression of *bax* in the 19K1 cell line, which undergoes p53-mediated growth arrest at the permissive temperature (Debbas and White 1993; Lin et al. 1995; Sabbatini et al. 1995a), was also up-regulated three- to fivefold at the permissive temperature (Fig. 6A). The 19K1 cell line is protected from apoptosis, and any reduction in intact mRNA attributable to cell death is therefore prevented, which may account for the prominent *rbax* mRNA induction. In contrast, *bax* mRNA levels were barely detectable in the 22-23val135A cell line at either the restrictive or permissive temperature, consistent with the fact that tsp53(22-23val135) is transcriptionally defective at both temperatures (Sabbatini et al. 1995b). Moreover, the patterns of expression of the p53-inducible gene *p21/Waf-1/Cip-1* and *rbax* in all cell lines were identical (Fig. 6A). These results are consistent with previous findings demonstrating that p53-mediated *trans*-activation remains intact in both the An1a and 19K1 cell lines but is dramatically impaired in the 22-23val135A cell line (Sabbatini et al. 1995b). Thus, apoptosis appears to require a transcriptionally competent p53, and *bax* mRNA induction parallels *p21/WAF-1/Cip-1* mRNA induction and correlates with apoptosis.

Western blot analysis indicates that rBax protein levels increased dramatically and progressively in both the An1a and An1b cell lines following incubation at the permissive temperature (Fig. 6B). Bax protein levels also increased progressively in both the 19K1 and 4B cell lines at the permissive temperature (Fig. 6B). The apparent slight difference in the kinetics of Bax accumulation in 19K1 and 4B compared to the An1 cell line was attributable to variations in blotting conditions, as it was not observed when An1, 19K1, and 4B were analyzed on the same blot (data not shown). Interestingly, Bax protein levels remained high in the 19K1 and Bcl-2 cell lines through 96 hr at the permissive temperature, at which point cells have undergone p53-mediated growth arrest (Fig. 6B). E1B 19K protein levels also increased dramatically (Fig. 6B). There was no increase in 19K mRNA levels, and in pulse–chase experiments, the half-life of the E1B 19K protein was increased from 4 hr to >12 hr (data not shown). Although E1B 19K protein accumulation was apparently the result of an increase in the stability of the protein, whether this was caused by the presence of rBax has not yet been determined. As predicted from the Northern blot analysis, rBax protein levels were barely detectable in the 22-23val135A cell line at either the restrictive or permissive temperature (Fig. 6B). In addition, the corresponding patterns of expression of rBax and p21/Waf-1/Cip-1 protein in each cell line were identical (Fig. 6B).

These results indicate that Bax and p21/Waf-1/Cip-1 are equally up-regulated in BRK cell lines that succumb to either p53-mediated apoptosis or growth arrest at the permissive temperature. In addition, they are consistent with the fact that the E1B 19K and Bcl-2 proteins function downstream of p53-mediated *trans*-activation to overcome p53-mediated apoptosis.

Bax is sufficient for apoptosis and acts downstream of p53

p53 can activate the transcription of genes involved in growth arrest, such as *p21/Waf-1/Cip-1*, and in apoptosis, such as *bax*. In BRK cells the induction of apoptosis requires a transcriptionally competent p53 (Sabbatini et al. 1995b). *bax* induction by p53 may be responsible for apoptosis, or *bax* may be only one gene product of many that is required for activation of the death pathway by p53. To test this hypothesis, rBax was transfected into the An1 cell line at the restrictive temperature where p53 was constitutively in the mutant conformation to determine whether *bax* expression alone was capable of inducing cell death.

An1 was transfected at 38.5°C (restrictive temperature) with the rBax expression vector (pCEP4–Myc–rBax) containing a hygromycin resistance marker or a vector control lacking the rBax cDNA and placed under drug selection. After 21 days, the number of hygromycin resistant colonies was evaluated by Giemsa staining. As seen in Fig. 7, there was a sixfold reduction in the number of colonies in the rBax-transfected cells compared with the control. Transient Bax expression induced cell death that resembled apoptosis (data not shown), suggesting that Bax was sufficient for the induction of apoptosis in the presence of mutant p53. Thus, Bax alone was capable of inducing apoptosis, which is consistent with it functioning downstream of p53.

Discussion

The adenovirus E1B 19K protein plays an important role in inhibiting apoptosis during the productive infection of human cells and during the transformation process of primary rodent cells. Without E1B 19K function, virus production is compromised because of premature death of the host cell (White 1994; White and Gooding 1994) and transformation by E1A is aborted (Rao et al. 1992; White et al. 1992). Early evidence suggested that the E1B 19K protein functioned as an apoptosis inhibitor and possessed both structural and functional homology to Bcl-2 (White et al. 1991; Rao et al. 1992; White et al. 1992). The capacity of the E1B 19K protein to interact and display functional antagonism with Bax further supports the suggestion that it is the adenovirus Bcl-2 homolog. Other DNA viruses harbor open reading frames with ho-

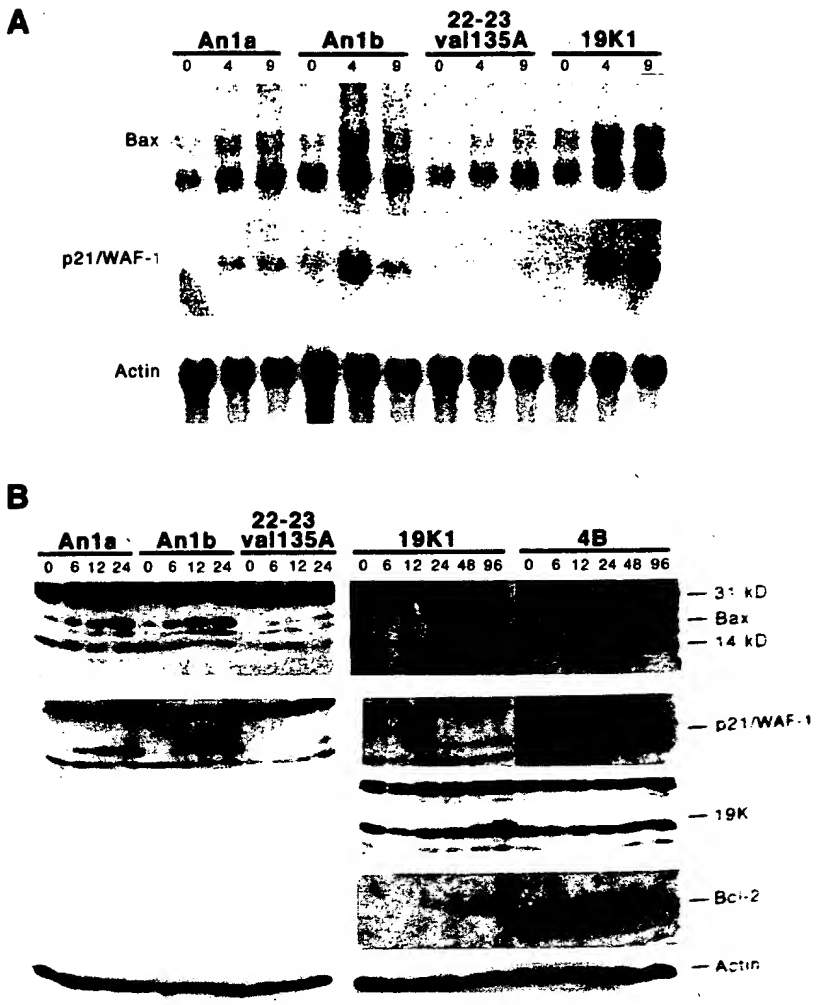


Figure 6. Up-regulation of *bax* and *p21/Waf-1/Cip-1* mRNA and protein levels in BRK cell lines by p53. (*A*) Northern blots of RNA isolated from the An1a, An1b, 22-23val135A, and 19K1 cell lines at the restrictive (0 hr) and permissive (4 and 9 hr) temperatures were hybridized with probes corresponding to *rbax* (*top*), murine *p21/Waf-1/Cip-1-1* (*middle*), and murine β-*actin* (*bottom*). (*B*) Western blot analysis of rBax and p21/Waf-1/Cip-1 expression in the An1a, An1b, 22-23val135A, 19K1, and 4B cell lines at the restrictive and permissive temperatures. Total cell extracts were prepared from the An1a, An1b, and 22-23val135A cell lines at the restrictive temperature (0 hr) and after incubation for 6, 12, and 24 hr at the permissive temperature. Extracts were prepared from the 19K1 and 4B cell lines at the restrictive temperature (0 hr) and after incubation for 6, 12, 24, 48, and 96 hr at the permissive temperature. Western blots were probed with antibodies specific for Bax (N-20), p21/Waf-1/Cip-1-1, and actin. Blots containing extracts from the 19K1 and 4B cell lines were also probed with antibodies directed against the E1B 19K (2F3) and Bcl-2 (1632-15) proteins, respectively.

mology to Bcl-2 (Cleary et al. 1986; Henderson et al. 1993; Neilan et al. 1993), whereas non-Bcl-2-related apoptosis inhibitors have been identified in baculovirus (Clem and Miller 1994) and poxviruses (Ray et al. 1992). It is becoming apparent that apoptosis is one cellular response to infection with a DNA virus and may represent an altruistic defense mechanism at the cellular level to contain or minimize virus infection of the host. Although the molecular events triggering apoptosis upon infection with other DNA viruses are obscure, with adenovirus the expression of the E1A proteins is responsible (White and Stillman 1987; White et al. 1991).

The ability of E1A to induce apoptosis is genetically inseparable from its ability to induce cellular DNA synthesis, suggesting that deregulation of cell growth control, either directly or indirectly, through the induction of DNA damage, is responsible for promoting cell death (White et al. 1991; Mymryk et al. 1994; Teodoro et al. 1995). In rodent cells the induction of apoptosis by E1A requires the function of the p53 tumor suppressor gene product (Debbas and White 1993), suggesting that p53 may function in response to abnormal cell cycle progression induced by E1A. The p53 protein accumulates fol-

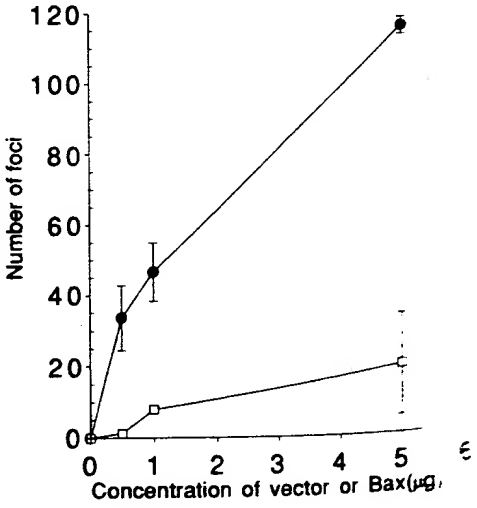
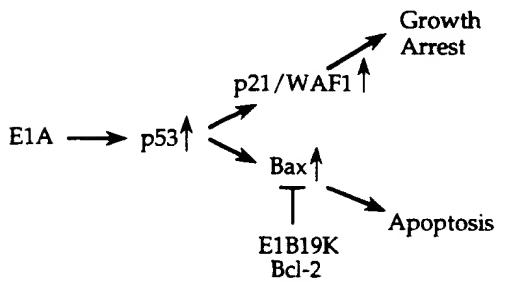


Figure 7. Bax is sufficient for induction of apopt... downstream of p53. The E1A plus tsp53-transform... line AN1 was transfected with 0.5, 1.0, and 5.0 μg... CMV–Bax plasmid (pCEP4–Myc–rBax) or control... (□) by electroporation. After 21 days post-transfect... with selection for hygromycin resistance (●), the f... number was determined following Giemsa stainin...

ring E1A expression (Lowe and Ruley 1993; Chiou et 1994a), which mimics the p53 response to DNA dam- (Maltzman and Czyzyk 1984; Kastan et al. 1991, 2; Kuerbitz et al. 1992), supporting a role for p53 as a veillance factor to induce growth arrest or apoptosis ibnormal, damaged, or infected cells. p53 can induce wth arrest or apoptosis in different physiological set- gs. Bcl-2 and E1B 19K expression can specifically ck the induction of apoptosis by p53, but the growth st function is intact (Debbas and White 1993; Chiou l. 1994a; Lin et al. 1995; Sabbatini et al. 1995a). This gests that growth arrest and apoptosis are distinct ivities of p53 and that Bcl-2 levels may be a determin- factor in regulating the physiological outcome fol- ring p53 induction.

53 is a transcription factor that activates the tran- ption of a number of cellular genes, one of which is *p21/Waf-1/Cip-1* cell cycle-dependent kinase inhib- (El-Deiry et al. 1993; Harper et al. 1993; Xiong et al. 3; Noda et al. 1994). p21 expression can induce cell le arrest, suggesting that it is one means by which can arrest cell growth. The role of the transcriptional vity of p53 in regulating apoptosis has been less clear may be cell type specific (Caelles et al. 1994; Haupt l. 1995; Sabbatini et al. 1995b). Transcriptional in- tion of the *bax* gene by p53, for example, has been ciated with apoptosis in some settings (Miyashita et 1994; Miyashita and Reed 1995) but not in others nman et al. 1995). In the E1A plus p53(val135) BRK isformants that we have generated, however, p53 re- res a functionally competent *trans*-activation domain the induction of apoptosis (Sabbatini et al. 1995b). is, the activity of p53 as a transcription factor is ly required for induction of apoptosis in BRK cells. could potentially activate the transcription of death es such as *bax* or repress the transcription of survival es such as *bcl-2* to promote cell death. Because the 19K and Bcl-2 proteins both associate with Bax, they ld potentially prevent any adverse effect of Bax on viability following p53-promoted Bax accumulation 8). *bax* mRNA and protein levels were induced spe- cally and dramatically upon conformational change 53 from the mutant to the wild-type form, and a iscriptionally defective p53 was incapable of display-



re 8. Model for separation of p53-mediated apoptosis and th arrest functions by interaction of the E1B 19K and Bcl-2 eins with the Bax protein.

ing this activity, providing strong evidence that *bax* is either directly or indirectly regulated by p53 at the transcriptional level.

E1B 19K and Bcl-2 expression, although sufficient to block cell death, had no impact on *bax* message or protein accumulation, suggesting that they act downstream of *bax* induction by p53 (Fig. 8). Furthermore, as the E1B 19K- and Bcl-2-expressing cells do not die, Bax induction by p53 can be uncoupled from cell death, ruling out the possibility that Bax induction is merely a symptom of cells undergoing the death process. E1B 19K protein levels increase following Bax induction. There was an increase in the half-life of the 19K protein but no change in the level of 19K message, suggesting a post-transcriptional mechanism of protein accumulation. Perhaps 19K stabilization is prompted by the sudden increase in and interaction with Bax.

Bax has been demonstrated to associate with Bcl-2 and antagonize the ability of Bcl-2 to block apoptosis (Oltvai et al. 1993). In functional assays, Bax expression also antagonized the ability of the E1B 19K protein to block apoptosis. E1B 19K and Bcl-2 expression in BRK cells has no measurable effect on cell viability or growth unless p53 is converted to the wild-type form. Only under these circumstances when Bax levels, which are virtually undetectable in the presence of mutant p53, are increased by the presence of wild-type p53, is any functional activity of either E1B 19K or Bcl-2 apparent. It is possible that Bax is ineffective at inducing apoptosis as long as Bcl-2 and E1B 19K proteins are expressed in relative excess of Bax and thus they may serve to sequester Bax activity to preserve cell viability.

Bax expression induced apoptosis in cells that constitutively expressed mutant p53, suggesting that Bax alone was sufficient to induce cell death and that it acts downstream of p53. Whether *bax* is the only death gene transcriptionally activated by p53, or one of many that function redundantly, is not yet known. When expressed in other cell types, however, Bax expression is not lethal and only induces apoptosis upon receipt of a death signal such as factor withdrawal (Oltvai et al. 1993). A death signal is apparently necessary in some cell types and may depend on the levels of expression of endogenous Bcl-2-like proteins. The induction of apoptosis by Bax in BRK cells in the presence of mutant p53 may be because the level of expression of endogenous Bcl-2-like proteins is low (both Bcl-2 and Bcl-*x* protein levels are barely detectable in BRK cell lines; data not shown). Alternatively, the E1A expressed in BRK cells may provide a constitutive death signal that only requires wild-type p53, and thereby Bax expression, for implementation of the death program.

The relationship between the cell cycle and apoptosis has been suggested by a number of experiments, but the molecular basis of the relationship has been unclear. Deregulation of cell growth by E1A and c-*Myc* expression can stimulate p53 accumulation and p53-dependent apoptosis (Askew et al. 1991; White et al. 1991; Evan et al. 1992; Amati et al. 1993; Hermeking and Eick 1994). Whether apoptosis is the result of conflicting growth sig-

nals generated by simultaneous opposing positive and negative forces governing cell cycle progression, or some other cause, is not known. Apoptosis has been reported to occur preferentially at the G_1/S transition and S in some cases (Yonish-Rouach et al. 1993), in any phase of the cell cycle in others, or to either precede or follow growth arrest. Our data suggest that p53 simultaneously activates both the growth arrest and apoptosis pathways through the transcriptional activation of *p21/Waf-1/Cip-1* and *bax* genes, respectively (Fig. 8). In situations of limiting amounts of Bcl-2 or E1B 19K proteins, the apoptotic pathway will be dominant over the growth arrest pathway. In Bcl-2 or E1B 19K protein excess (and Bax sequestration), the growth arrest pathway becomes apparent because apoptosis is inhibited (Fig. 8). The relationship between apoptosis and the cell cycle in this situation may be limited to the causation of p53 accumulation, perhaps because of the generation of DNA damage through aberrant or unregulated DNA synthesis. Thus, with the simultaneous activation of both growth arrest and apoptosis pathways, apoptosis is dominant over growth arrest (Fig. 8). This model would be consistent with the observation that Bcl-2 and E1B 19K expression specifically block p53-dependent apoptosis while preserving the growth arrest function of p53.

The E1B 19K protein bears a strong resemblance to Bcl-2 both in functional assays and in amino acid sequence. The BH1 region in particular is highly conserved in the E1B 19K proteins encoded by the 12 adenovirus serotypes for which there is sequence information (Chiou et al. 1994b). The invariant glycine at codon 87 in the adenovirus 2 (Ad2) E1B 19K protein is also invariant among members of the Bcl-2 family. Glycine-to-alanine substitution at this position causes a loss of the ability to inhibit apoptosis in both the E1B 19K and Bcl-2 proteins (White et al. 1992; Chiou et al. 1994b; Yin et al. 1994). Interestingly, this same amino acid substitution in either the E1B 19K or Bcl-2 protein prevents interaction with Bax, suggesting a common biochemical mechanism of action. What was surprising was the identification of a third conserved region, BH3, that is sufficient for the interaction of Bax with either E1B 19K or Bcl-2. In contrast, the corresponding E1B 19K BH3 is required but not sufficient for interaction with Bax. The central conserved domain encompassing BH1, BH2, and BH3 of E1B 19K is the minimal region of E1B 19K sufficient for interaction with Bax BH3 (amino acids 50–78). When the region of the E1B 19K protein that is sufficient for association with full-length rBax was mapped, however, 19K BH3 (amino acids 19–57) could interact weakly, but 19K BH3 and rBax BH3 did not interact. Thus, the BH3 region of either 19K or Bax may be an interaction domain for association with multiple domains (BH1–BH3) on the opposite binding partner. Nonsymmetrical interactions between the E1B 19K or Bcl-2 and Bax proteins are therefore, likely. Participation of regions outside of BH1 and BH2 in interactions between Bcl-2 family members was also suggested by the interaction between Bcl-x_S and Bcl-x_L. Bcl-x_S, which is a product of a splice variant of the *bcl-x* gene, is missing BH1 and BH2 but can interact with and antagonize the function of another alternatively spliced product, Bcl-x_L, which retains BH1 and BH2 (Boise et al. 1993; Sato et al. 1994). Perhaps BH3 of Bcl-x_S confers the ability to interact with BH1–BH3 of Bcl-x_L, as is the case of the interaction between Bax and E1B 19K.

The identification of such a small binding region (BH3) that is sufficient for the interaction of Bax with either the E1B 19K or Bcl-2 proteins, or for interaction of 19K with Bax, may account for the large number of different proteins that have been pulled out of yeast two-hybrid assays and expression cloning for interaction with members of the Bcl-2 family (Boyd et al. 1994; Farrow et al. 1995; Takayama et al. 1995; Yang et al. 1995). At least one other 19K-binding protein (BP4) that we isolated from the two-hybrid screen has sequence homology with BH3 (J. Han and E. White, unpubl.). Whether this region is responsible for 19K association or this protein has any role in the regulation of apoptosis, remains to be determined. It is conceivable that such a short sequence (<29 amino acids) may be represented in multiple proteins that may or may not be relevant for apoptosis. Determination that proteins interact with members of the Bcl-2 family in vivo and that the interaction has some physiological relevance may therefore be imperative.

Another Bcl-2 family member, Bak, has been shown to interact with the 19K protein in yeast two-hybrid assays and may act similarly to Bax as an apoptosis regulator (Chittenden et al. 1995a; Farrow et al. 1995; Kiefer et al. 1995). Bak contains the conserved BH3 region but it is not yet known if the binding requirements for 19K and Bak are the same as those for 19K and Bax. Bak is not detectably expressed in BRK cells, whereas Bax is, suggesting that the 19K–Bak interaction may not be physiologically relevant in this cell type but may be important in others.

Bak, like Bax, has a BH3 domain (Fig. 1C). Analysis of Bak deletion mutants has shown that retention of the BH3 domain is essential for the promotion of apoptosis and for interaction with Bcl-x_L (Chittenden et al. 1995b). Furthermore, expression of a 50-amino-acid segment of Bak, including BH3 but excluding BH1 and BH2, was sufficient for the induction of apoptosis (Chittenden et al. 1995b). Thus, the BH3 domain and sequences in the immediate vicinity encode the apoptosis-promoting activity that may act by either interacting with and interfering with a Bcl-2-like activity or through possession of an effector activity. Presumably Bax functions analogously.

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s and methods

id system

DNA library in the pGAD–GH vector (Hannon et al.
screened in the yeast strain YGH1 (*ura3-25, his3-200, lys-2-801, trp1-901, leu2-3*, Can^r, *gal4-542, gal80-538,* 1_{uas}–*gal1*$_{tata}$–*HIS3, URA3::gal1–lacZ*). The pGBT-9–
nid was cotransfected with the pGAD library plasmid
)dard lithium acetate transfection procedures. Trans-
were plated on yeast dropout plates lacking leucine,
n, and histidine. Approximately 3 × 10^6 transfor-
re screened for growth in the absence of histidine and
)r β-galactosidase activity, as a second reporter, using
)sed assay (Hannon et al. 1993). The library plasmid
tively recovered by transforming yeast mini prep DNA
erichia coli MH4 which is *leuB*$^-$ and therefore selects
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)lification and cloning

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et al. 1995; White et al. 1986) and reverse transcribed
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(GIBCO–BRL). To clone rat Baxα, the complementary DNA was resuspended in a total volume of 100 μl containing 2.5 units of *Taq* DNA polymerase (Perkin-Elmer), 20 μM each of dGTP, dATP, dTTP, and dCTP, 50 pmoles of forward primer (5′-TAATACCCGGGTATGGACGGGTCCGGGGAGC-3′) and reverse primer (5′-CGCTGCTCGAGTCAGCCCATCTTCTTC-CAGAT-3′), and 1× reaction buffer. Thirty-five amplification cycles consisted of denaturation at 94°C for 1 min (first cycle 4 min); annealing at 58°C for 1 min; and extension at 72°C for 2 min. PCR products were size fractionated by 1.2% agarose electrophoresis, and the expected 598-bp product was purified, digested with *Xma*I and *Xho*I, and ligated into the pGAD–GH vector. The insert was sequenced to verify that it encoded rBax. The amino acid sequence of rBax had 91.7% identity (95.3% similarity) with full length hBax and 93.1% identity (96.6% similarity) with BH3 of hBax.

Plasmid construction

A 552-bp fragment containing the wild-type E1B 19K open reading frame was PCR cloned from the pCMV19K expression vector (White and Cipriani 1989) into the pGBT-9 plasmid in frame with the DNA-binding domain of GAL4. A specific 5′ primer was used to engineer an *Eco*RI site before the ATG and the M13 reverse primer served as the 3′ primer. The fragment was cloned into the *Eco*RI and *Bam*HI sites of the pGBT9 vector. Standard PCR reactions were used to construct yeast fusion plasmids (Bp3–Ex3/4′ in pGAD–GH and Bp3–Ex3 in pGAD–GH). The PCR products were digested with *Xma*I and *Xho*I and ligated in pGAD–GH.

cDNAs encoding the E1B 19K missense mutants pm7, pm44, pm51, pm87, and pm102 (White et al. 1992; Chiou et al. 1994b) were digested with *Eco*RI and *Bam*HI. The 552-bp E1B 19K mutant open reading frames were then cloned in frame with the DNA-binding domain of pGBT9. Standard PCR techniques were used to construct the E1B 19K deletion mutants. The primers were designed to include an *Eco*RI site at the 5′ end and a *Bam*HI site at the 3′ end to clone the fragments in-frame with the DNA-binding domain of the pGBT-9 vector.

To produce the rBax mammalian expression vector pCEP4–Myc–rBax, oligonucleotides encoding a Myc epitope tag with flanking *Kpn*I and *Xma*I sites were annealed with the *rbax* cDNA containing flanking *Xma*I and *Xho*I sites and ligated into the pCEP4 vector that had been digested with *Kpn*I and *Xho*I. This placed the Myc epitope tag in frame at the amino terminus of rBax. The E1B 19K expression vector pcDNA3-19K was constructed by ligating the *19K* coding region into the *Hin*dIII site of pcDNA3. The orientation of the insert was confirmed by digestion with *Kpn*I. Myc–rBax was also cloned into the *Kpn*I and *Xho*I sites of pcDNA3 to generate the pT7–Myc–rBax vector for in vitro transcription and translation of Bax. The human *bcl-2* under the control of the *T7* promoter was constructed by cloning *bcl-2* into the *Eco*RI site of pGEM-2 to generate pGEM-2–Bcl-2 (kindly provided by Dr. C. Gelinas, University of Medicine and Dentistry of New Jersey. (UMDNJ)). The E1B 19K open reading frame and the 19K mutant pm51 were subcloned from their respective pGBT-9 plasmids by restriction digestion with the *Eco*RI and *Sal*I into the pGEX–4T1 plasmid to generate pGST19K and pGSTpm51. The 5′ *Eco*RI site maintained the frame to read through into a GST fusion protein. For construction of the pT719K expression vector, a unique *Nde*I site was created at the ATG of the E1B 19K open reading frame by site-directed mutagenesis. A fragment containing the 19K open reading frame (nucleotides 1711–2256 of the adenovirus genome) was then subcloned into a T7 promoter expression vector (generously provided by Drs. O. W. Studier and J. Dunn, Brookhaven National Laboratory, NY). The pT719K construct also contained a stop codon in the overlapping E1B 55K reading

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nine-labeled extracts, a protein that comigrates with Bax is virtually the only protein that was specifically isolated based on the ability to interact with the 19K protein. Thus, the interaction between the E1B 19K and Bax proteins is highly specific and can occur in the complex milieu of a crude cell lysate. The 19K protein is highly insoluble and is only liberated by the presence of ionic detergents that would normally destroy protein–protein interactions (White et al. 1984; White and Cipriani 1990). We have therefore not been able to coimmunoprecipitate E1B 19K with Bax (or any other protein) in cell lysates. The GST–19K pulldown assays with whole cell extracts as a source of Bax may be the only means to evaluate the potential for an in vivo interaction.

In BRK cells Bax appears to be sufficient for the induction of apoptosis. By binding to and neutralizing Bax, the E1B 19K and Bcl-2 proteins could act as apoptosis inhibitors. E1B 19K and Bcl-2 expression have no measurable effect on cell growth or viability, but their activity becomes apparent when Bax is present, suggesting that Bax is the effector of cell death. The functional outcome of Bax and Bcl-2 expression (cell death vs. protection from cell death), however, may depend on the presence of other Bcl-2 family members and the nature of the interactions between them. Presumably, the Bcl-2 family acts either directly or indirectly as regulators of the ICE family, and it will be of interest to determine which cysteine protease is activated by Bax or inhibited by E1B 19K and Bcl-2. Finally, defining the specific interaction domains such as BH3 may identify new targets for anti-cancer therapy.

Materials and methods

Two-hybrid system

A HeLa cDNA library in the pGAD–GH vector (Hannon et al. 1993) was screened in the yeast strain YGH1 (*ura3-25, his3-200, ade2-101, lys-2-801, trp1-901, leu2-3,* Can^r, *gal4-542, gal80-538, LYS2::gal1*$_{uas}$*–gal1*$_{tata}$*–HIS3, URA3::gal1–lacZ*). The pGBT-9–19K plasmid was cotransfected with the pGAD library plasmid using standard lithium acetate transfection procedures. Transformants were plated on yeast dropout plates lacking leucine, tryptophan, and histidine. Approximately 3×10^6 transformants were screened for growth in the absence of histidine and assayed for β-galactosidase activity, as a second reporter, using a filter-based assay (Hannon et al. 1993). The library plasmid was selectively recovered by transforming yeast mini prep DNA into *Escherichia coli* MH4 which is *leuB*$^-$ and therefore selects for the leucine metabolic marker on the pGAD plasmid (Hannon et al. 1993). False-positives were eliminated by screening for interaction with irrelevant proteins (Apc-2) and the empty pGBT-9 vector. Sequence analysis of the cDNAs encoding the interacting proteins was performed using Sequenase 2.0 (U.S. Biochemical) according to the manufacturer's specifications.

PCR amplification and cloning

Cytoplasmic RNA was extracted from the An1 cell lines after incubation for 4 hr at 32°C using previously described methods (Sabbatini et al. 1995; White et al. 1986) and reverse transcribed with Moloney murine lymphotrophic virus reverse transcriptase (GIBCO–BRL). To clone rat Baxα, the complementary DNA was resuspended in a total volume of 100 μl containing 2.5 units of *Taq* DNA polymerase (Perkin-Elmer), 20 μM each of dGTP, dATP, dTTP, and dCTP, 50 pmoles of forward primer (5′-TAATACCCGGGTATGGACGGGTCCGGGGAGC-3′) and reverse primer (5′-CGCTGCTCGAGTCAGCCCATCTTCTTC-CAGAT-3′), and 1× reaction buffer. Thirty-five amplification cycles consisted of denaturation at 94°C for 1 min (first cycle 4 min); annealing at 58°C for 1 min; and extension at 72°C for 2 min. PCR products were size fractionated by 1.2% agarose electrophoresis, and the expected 598-bp product was purified, digested with *Xma*I and *Xho*I, and ligated into the pGAD–GH vector. The insert was sequenced to verify that it encoded rBax. The amino acid sequence of rBax had 91.7% identity (95.3% similarity) with full length hBax and 93.1% identity (96.6% similarity) with BH3 of hBax.

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frame from the pMT19K construct (White and Cipriani 1989) to prevent expression of other E1B products.

Antibodies

Monoclonal antibodies directed against the E1B 19K protein were generated by immunizing mice with full-length E1B 19K protein purified from *E. coli*. The pT719K vector was transformed into the BL21 DE3 *E. coli* strain, and the 19K protein was purified by standard chromatographic procedures. The purified protein was used to immunize BALB/c mice, and hybridomas were generated by standard protocols (Harlow and Lane 1988). The 19K-specific hybridoma 2F3 was injected into mice to obtain ascites fluid. Polyclonal antibodies directed against Bax (N-20) and Bcl-2 (ΔC-21) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The monoclonal Myc antibody-1 was purchased from Oncogene Science, Inc. (Cambridge, MA). A polyclonal antibody directed against the E1B 19K protein (p21) and a monoclonal antibody directed against the E1B 55K protein (13D2) have been described previously (White and Cipriani 1989; White and Cipriani 1990). The monoclonal antibody directed against murine p21/Waf-1/Cip-1 was a kind gift from Dr. David Hill of Oncogene Science, Inc. (Cambridge, MA). An anti-actin monoclonal antibody was purchased from Amersham Corp. (Arlington, IL). An anti-rBcl-2 (1632-15) polyclonal antibody that also cross-reacts with hBcl-2 was kindly provided by Dr. J. Reed (La Jolla Cancer Research Foundation, La Jolla, CA).

In vitro protein interaction assays

mRNAs encoding human Bcl-2 (pGEM-2–Bcl-2), E1B 19K (pcDNA3–19K), and rBax (pcDNA3–rBax) were prepared by in vitro transcription. mRNAs were titrated to generate equal quantities of translated protein products. The in vitro transcription and translation were performed using a commercial kit (Promega). The ^{35}S-labeled proteins were incubated for 2 hr with anti-Bcl-2 (ΔC21), anti-Bax (N-20), or anti-19K (p21) antibodies in immunoprecipitation buffer [50 mM Tris (pH 7.5), 150 mM NaCl, and 0.2% NP-40] and washed three times with the same buffer containing 0.01% NP-40. The immunoprecipitated proteins were analyzed on 17% SDS gels. Gels were fixed in 50% methanol and 10% glacial acetic acid for 2 hr, dried, and scanned using a PhosphorImager (Molecular Dynamics).

For fusion protein binding assays, plasmids were transformed into BL21 DE3 and induced with 0.5 mM of isopropyl-β-D-thiogalactoside. A 50-μl aliquot of culture was analyzed by SDS-PAGE to evaluate fusion protein induction. The remaining culture was sonicated on ice by using 10 short bursts at 10 sec each (Fischer Sonic Dismembranator 3000), clarified by centrifugation, and the supernatant was resuspended in 50% (vol/vol) glutathione–Sepharose beads (Pharmacia Biotech, Piscataway, NJ). An aliquot of the protein bound beads was analyzed by SDS-PAGE to ensure equal amounts of pure fusion protein were present.

In vitro binding assays were performed by incubating equal amounts of GST, GST–19K, and GST–pm51 (see Fig. 3B), immobilized onto glutathione–Sepharose beads, with in vitro-translated rBax or hBcl-2 proteins diluted in 0.5 ml of NETN buffer [20 mM Tris (pH 8.0), 100 mM NaCl, 1 mM EDTA, 0.2% NP-40]. The mixture was incubated for 2 hr at 4°C, washed three times with NETN buffer, and resuspended in 2× Laemmli buffer. rBax and hBcl-2 proteins translated in vitro were also immunoprecipitated with anti-Myc, anti-Bcl-2, or anti-E1B 55K antibodies to determine the relative levels of protein production. All samples were boiled for 5 min, and proteins were resolved by 15% SDS-PAGE and analyzed as described above.

For detection of the E1B 19K and rBax interaction in cell lysates, the E1A plus tsp53(val135)-transformed cell line An1 (Debbas and White 1993) was plated at 95% confluence and incubated at the permissive temperature (32°C) for 14 hr. Cells were washed with PBS and lysed in 1 ml of cold NETN lysis buffer containing protease inhibitors (0.1 mM phenymethyl sulfonylfluoride, 10 mM benzamidine, 0.1 mg/ml of bacitracin, 1.0 μg/ml of pepstatin A, 10 mM sodium bisulfite) for 20 min. Cells were centrifuged at 10K for 10 min to remove cellular debris. The lysates were then incubated with GST, GST–19K, and GST–pm51 proteins bound to glutathione–Sepharose beads for 2 hr and washed as described above. Samples were resolved by 15% SDS-PAGE. rBax was detected by Western blot analysis using a Bax-specific polyclonal antibody (N-20).

Binding assays with labeled cell extracts were performed by incubating the An1 cell line at 32°C for 12 hr, with methionine-deficient media in the presence of [^{35}S]methionine for 4 hr. The cells were lysed in NETN buffer and incubated with GST and GST–19K fusion proteins and washed, as described above. Samples were resolved by SDS-PAGE and fixed and fluorographed by standard procedures.

Indirect immunofluorescence

19K1, the E1A plus tsp53(val135)-transformed BRK line that expresses the E1B 19K protein (Debbas and White 1993), was plated at 90% confluence at the restrictive temperature (38.5°C) and incubated at 32°C for 72 hr. The 19K1 cell line was electroporated with 10 μg of the CMV Myc-tagged Bax expression vector (pCEP4–Myc–rBax) and incubated at 32°C. Cells were fixed with methanol 48 hr post-transfection and double-labeled with an anti-Myc monoclonal antibody at a 1:5 dilution and an anti-19K polyclonal antibody at a 1:200 dilution. Antibodies were visualized with goat anti-mouse rhodamine-conjugated and goat anti-rabbit fluorescein-conjugated secondary antibodies.

A TdT assay (Gavrieli et al. 1992) was utilized as an indicator of apoptotic cell death. 19K1 cells grown on coverslips were incubated at 38.5°C and transfected with 20 μg of either pCEP4 vector or pCEP4–Myc–rBax and then incubated for an additional 48 hr to permit Bax protein expression. The cells were then fixed with 2% paraformaldehyde in PBS followed by 70% ethanol and incubated with biotinylated dUTP and TdT (0.3 enzymatic units/ml, Boehringer Mannheim Biochemicals) as described previously (Gavrieli et al. 1992). Cells were washed with 0.125% BSA in PBS, incubated with avidin–FITC, and visualized by epifluorescence microscopy.

Bax functional assays

To examine Bax function in transient expression assays, the pcDNA3–19K construct was cotransfected with pCEP4–Myc–rBax into the An1 cell line by electroporation as described previously (Chiou et al. 1994a). The amount of transfected pcDNA3–19K DNA was fixed at 10 μg, and was cotransfected with 1, 5, 10, and 20 μg of control vector or pCEP4–Myc–rBax DNA. The An1 cell line was also transfected with pCEP4–Myc–rBax vector alone at the same concentration. The transfected cells were incubated at 38.5°C for 60 hr and shifted to 32°C for 60 hr. The viable cell number was assessed by trypan blue exclusion. For colony formation assays, the An1 cell line was transfected with 0.5, 1, and 5 μg of linerized pCEP4–Myc–rBax plasmid by electroporation. Stable cell lines were maintained at 38.5°C and selected by the addition of hygromycin to the me-

dium (2×10^6 U/ml of medium), and the foci number was determined by Giemsa staining at 21 days post-transfection.

Northern blot analysis

Cytoplasmic RNA was extracted from cell lines at the restrictive temperature (38.5°C, 0-hour time point) and after 4 and 9 hr incubation at the permissive temperature (32°C) using methods described previously (Sabbatini et al. 1995b). Twenty micrograms of cytoplasmic RNA from each cell line was subjected to Northern blot analysis. The probes used for Northern blot analysis were an oligonucleotide (5′-CTGCAGCTCCATATTGCTGTCCAGTTCATCTCCAATTCGCCGGAG-3′) specific for a region within exon 3 of *rbax*, and full-length cDNAs corresponding to mouse *p21/Waf-1/Cip-1*, and β-*actin*. Northern blots were scanned using a PhosphorImager (Molecular Dynamics).

Western blotting

Cell extracts for Western blot analysis were prepared from subconfluent cultures, and 80 μg of protein from each cell line was analyzed by SDS-PAGE and semidry blotting onto nitrocellulose membranes by standard procedures. Immune complexes were detected by enhanced chemiluminescence (ECL) according to the manufacturer's specifications (Amersham).

Ackn wledgments

We thank Drs. Linda Van Aelst, Maria Fernandez-Sarabia, and Jim Bishoff for providing the HeLa cell cDNA library in pGAD–GH, Dr. Céline Gélinas for providing human *bcl-2* in pGEM-2, Dr. P. Verwaerde for constructing the pGBT9–19K vector, and Dr. Diane Harvey for construction of the pT719K expression vector. We also thank Dr. John Reed for providing a Bcl-2 antibody and Drs. S. Chiou and G. Kasof for helpful comments and suggestions. This work was supported by grants from The National Institutes of Health (CA53370 and CA60088) to E.W. P.S. was supported in part by a Center for Advanced Biotechnology and Medicine (CABM) predoctoral fellowship.

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Oncogene (1998) 16, 1587–1591

Overexpression of *Bax* gene sensitizes K562 erythroleukemia cells to apoptosis induced by selective chemotherapeutic agents

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***Bax* and *Bcl-2* are a pair of important genes that control programmed cell death, or apoptosis, with *Bax* being the apoptosis promoter and *Bcl-2* the apoptosis protector. Although the detailed mechanism is unknown, the protein products of these two genes form protein dimers with each other and the relative ratio of the two proteins is believed to be a determinant of the balance between life and death. In our preliminary study, we found that K562 erythroleukemia cells have an extremely low level of endogenous Bcl-2 expression and a fairly high level of endogenous Bax expression. We constructed *Bax* and *Bcl-2* expression vectors and transfected them into K562 cells. We found that transfection of *Bax* vector increased the expression of Bax protein; a shortened form of Bax also appeared. Cell death analysis using the Annexin V assay showed that the *Bax* vector caused significantly more apoptotic cells that the *Bcl-2* or pCI-neo vector did. After selection with G418, *Bax*, *Bcl-2* and pCI-neo stably transfected cells were established. These three cell lines were examined for their response to the chemotherapeutic agents ara-C, doxorubicin, etoposide and SN-38. Bax-K562 cells showed significantly higher fractions of apoptotic cells than pCI-neo-K562 cells when treated with ara-C, doxorubicin or SN-38. No sensitization effect was seen when etoposide was used. In contrast, Bcl-2-K562 cells had fewer apoptotic cells than pCI-neo-K562 cells after treatment with all these agents. Therefore, Bax may sensitize K562 cells to apoptosis induced by a wide range of, but not all, chemotherapeutic agents.**

Keywords: Bax; Bcl-2; apoptosis; K562; chemotherapeutic agents

Introduction

The efficacy of chemotherapeutic agents depends on their effectiveness in inducing apoptosis of tumor cells. Different tumor cells respond to different chemotherapeutic agents in different ways. Understanding the mechanisms that underlie these different responses will aid in the design of specific therapy regimens. Studies of the last 10 years have identified the Bcl-2 family as a key regulator of cell death (Yang and Korsmeyer, 1996). The Bcl-2 family includes a series of homologous genes, such as *Bax*, *Bcl-2*, *Bcl-X_L*, *Bad* and others. The members of the Bcl-2 family have yin-yang roles in apoptosis regulation. These is strong evidence that Bcl-2 represses apoptosis induced by radiation (Strasser *et al.*, 1994), various chemotherapeutic agents (Ibrado *et al.*, 1996; Zhang *et al.*, 1996; Yin and Schimke, 1996), cytokine depletion (Oltvai *et al.*, 1993) and other cytotoxic conditions. It has been shown that Bax homodimerizes with itself and that a Bax/Bax dimer accelerates programmed cell death under some conditions, such as during interleukin-3 (IL-3) depletion (Oltvai *et al.*, 1993). Recently, using a reverse tetracycline-inducible system, it was shown that *Bax* expression induced apoptosis in Jurkat cells (Xian *et al.*, 1996). Members with opposite functions regulate each other by forming complexes with a new function. Therefore, the ratio of the levels of Bax and Bcl-2 is more important than the steady-state level of each individual protein in the cells. However, the exact roles played by the Bax protein are rather difficult to define, perhaps because Bax also forms complexes with other cellular proteins and because different cells may tolerate different steady-state levels of Bax protein. Along this line, it has been shown that Bax protein is expressed in many normal tissues (Krajewski *et al.*, 1994) and that *Bax*-transfected stable cell lines can be established (Oltvai *et al.*, 1993). Therefore, it is important to know whether Bax can induce apoptosis in cell lines that already express high levels of endogenous Bax.

Bax may also affect the response of cancer cells to chemotherapeutic agents and the clinical prognosis of cancer patients. Reduced expression of Bax was found to be associated with poor response rates to combination chemotherapy and shorter survival in women who had metastatic breast cancer (Krajewski *et al.*, 1995). Overexpression of Bax sensitized human breast cancer cells to radiation-induced (Sakakura *et al.*, 1996) and epirubicin-induced (Wagener *et al.*, 1996) apoptosis. Another study showed that Bax enhanced apoptosis in ovarian cancer cell lines, which were transfected with *Bax* gene expression vector, after treatment with paclitaxel, vincristine, or doxorubicin (Strobel *et al.*, 1996).

In this study, we investigated the role of Bax and Bcl-2 in the response of K562 erythroleukemia cells to cell death induced by a series of chemotherapeutic agents. The results suggest that Bax can selectively sensitize K562 cells to cell death induced by some chemotherapeutic agents. Bcl-2 protects these cells from apoptosis induced by all the agents used

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Results

Bax and Bcl-2 protein expression in transfected K562 cells

In a Western blotting survey of expression levels of Bax and Bcl-2 proteins in a series of leukemia cell lines, we found that Bcl-2 protein expression was very low in erythroleukemia K562 cells. In contrast, Bax protein was expressed at a high level (Figure 1). This expression pattern is rather intriguing to us because K562 cells are relatively resistant to apoptosis, which is not consistent with the cells' low levels of apoptosis-inhibiting Bcl-2 and high levels of apoptosis-facilitating Bax. In view of the findings that Bax and Bak (another Bcl-2 analogue) can inhibit apoptosis under different conditions and in different cell lines (Reed, 1997), there is a possibility that in K562 Bax may have an apoptosis-inhibitory function. However, this expression pattern does not necessarily argue against the traditional roles of Bax and Bcl-2 in K562 cells: many other factors may determine the threshold steady-state levels that would affect apoptotic response. For example, K562 cells are p53-null and express the *bcr-abl* oncogene, which was shown to inhibit apoptosis (Zhang *et al.*, 1994; McGahon *et al.*, 1994). To demonstrate the roles of Bax and Bcl-2 in the apoptosis response of K562 cells, we decide to use a gene transfer technique to modulate the expression of Bax and Bcl-2 and analyse for cell death in the presence and absence of additional death stimuli. *Bax* and *Bcl-2* cDNAs were cloned into the expression vector pCI-neo, and *Bax*, *Bcl-2* and pCI-neo control expression vectors were then transfected into K562 cells by electroporation. The cells killed by electroporation were removed by Ficoll separation 4 h after electroporation. Under our electroporation condition, 40–50% of cells are routinely transfected. Two days after electroporation, Bax and Bcl-2 proteins were analysed by Western blotting. In *Bax*-transfected K562 cells, the original form of Bax, which we named Bax(L), increased, and a smaller form of Bax, which we named Bax(S), appeared. The appearance of Bax(S) is very interesting because our previous studies showed that this form of Bax was associated with apoptosis in three leukemia cell lines (Kobayashi *et al.*, submitted). Bcl-2 protein expression was very low in K562 cells but was clearly detected in *Bcl-2*-transfected cells (Figure 1).

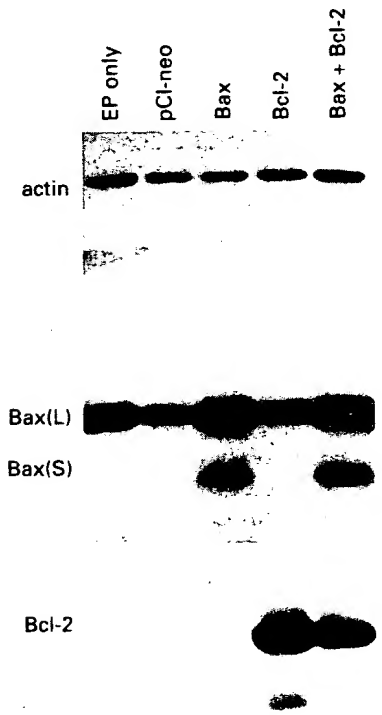

Figure 1 Expression of Bax and Bcl-2 proteins in transfected K562 cells. K562 cells were transfected with *Bax*, *Bcl-2* expression vectors or pCI-neo parental vector by electroporation. As a control, K562 cells were subjected to electroporation without DNA. Forty-eight hours after electroporation, total protein was isolated and 20 μg from each sample was analysed for expression of Bax and Bcl-2 on a Western blot. The blot was also analysed for expression of actin protein as a control for protein loading. Bax(L) migrates as a 21 kDa protein and Bax(S) migrates as a 18 kDa protein. EP, electroporation

Bax modestly induced apoptosis in K562 cells

The *Bax*-, *Bcl-2*-, and pCI-neo-transfected cells were analysed for apoptosis. The Annexin V assay was used because of its simplicity and sensitivity in detecting early stage apoptosis (Koopman *et al.*, 1994; Martin *et al.*, 1995; Boersma *et al.*, 1996; van Engeland *et al.*, 1996). In this assay, annexin V-FITC binds to phosphatidylserine, which during apoptosis is externalized to the outer cell membrane and is recognizable on the cell membrane of only apoptotic cells. The results showed that electroporation itself caused background levels of apoptotic cells and that pCI-neo vector and *Bcl-2* vector transfection resulted in only slightly higher levels of apoptotic cells, probably because of the toxic effect of DNA on the cells. In contrast, *Bax*-transfected cells had twice as many apoptotic cells as cells transfected by *Bcl-2* vector or pCI-neo control vector (Figure 2). The differences in the numbers of apoptotic cells were statistically significant. Therefore, elevated Bax expression can induce increased apoptosis in K562

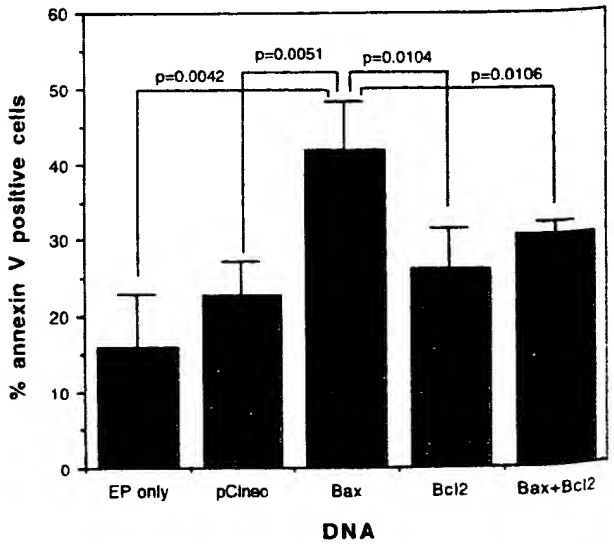

Figure 2 Bax induces apoptosis when transfected into K562 cells. K562 cells were electroporated with pCI-neo, Bax, Bcl-2, Bax + Bcl-2 or without DNA (EP only). cells killed by electroporation were removed by Ficoll after 4 h. The remaining cells were cultured for additional 48 h and analysed for apoptosis by Annexin V assays. The results are derived from three independent experiments

lls. However, the results also showed that Bax was ot a very potent promoter of apoptosis: less than 50% *Bax*-transfected cells were apoptotic. The results so showed that *Bcl-2*, when cotransfected with *Bax* pression vector, inhibited Bax-induced apoptosis. We so performed TUNEL apoptosis assays in selected periments. Similar results were obtained (data not own).

x sensitizes K562 cells to apoptosis induced by a series chemotherapeutic agents

nsistent with Bax being a weak cell death promoter r K562 cells, *Bax* stably transfected cells were tablished after G418 selection. Western blotting owed that the Bax(S) was retained in the stable cell e. *Bcl-2*- and pCI-neo-transfected cells were also tablished. These cells allowed us to examine whether x can sensitize K562 cells to chemotherapeutic ent-induced apoptosis. The *Bax*-, *Bcl-2*- and pCI-o-transfected K562 cells were treated with 2 μM ara-0.4 μg/ml doxorubicin, 20 μg/ml etoposide or 2 μM -38 (topoisomerase type I inhibitor) continuously 3 days. On each day of experiment, cells were taken m the culture and analysed for apoptosis by nexin V assay. The results in Figure 3 show that -transfected cells were more sensitive to ara-C-, xorubicin-, and SN-38-induced cell death than were -neo-transfected cells. However, *Bax*-transfected s were not more sensitized to etoposide. Bcl-2 erexpression repressed apoptosis in all treatments used here compared with pCI-neo control vector-transfected K562 cells. Bcl-2-K562 cells also had a lower percentage of spontaneously apoptotic cells compared with Bax-K562 and pCI-neo-K562 cells. We should point out that the cells we used in these experiments were not individual clones but total populations obtained after G418 selection. Therefore, the results reflect a true population-average phenomenon. The results were also highly reproducible, and except in one instance, differences in the levels of apoptosis were statistically significant.

Discussion

Since the Bcl-2 family was discovered, identification of its many members has snowballed. These members interact with each other and regulate the important cellular process of apoptosis involved in tissue development, homeostasis and tumor cell response to chemotherapy and radiotherapy (Yang and Korsmeyer, 1996). In this study, we investigated the roles of Bax and Bcl-2 in an erythroleukemia cell line, K562, which is p53-null. We were interested in this cell line because K562 cells have very low levels of endogenous Bcl-2 and abundant expression of endogenous Bax protein, despite the cells' noted resistance to many chemotherapeutic agents and radiation (McGahon *et al*., 1994; Kobayashi *et al*., 1995). Although the lack of p53 and presence of *bcr–abl* may contribute to the highly resistant nature of K562 cells, it is also possible that

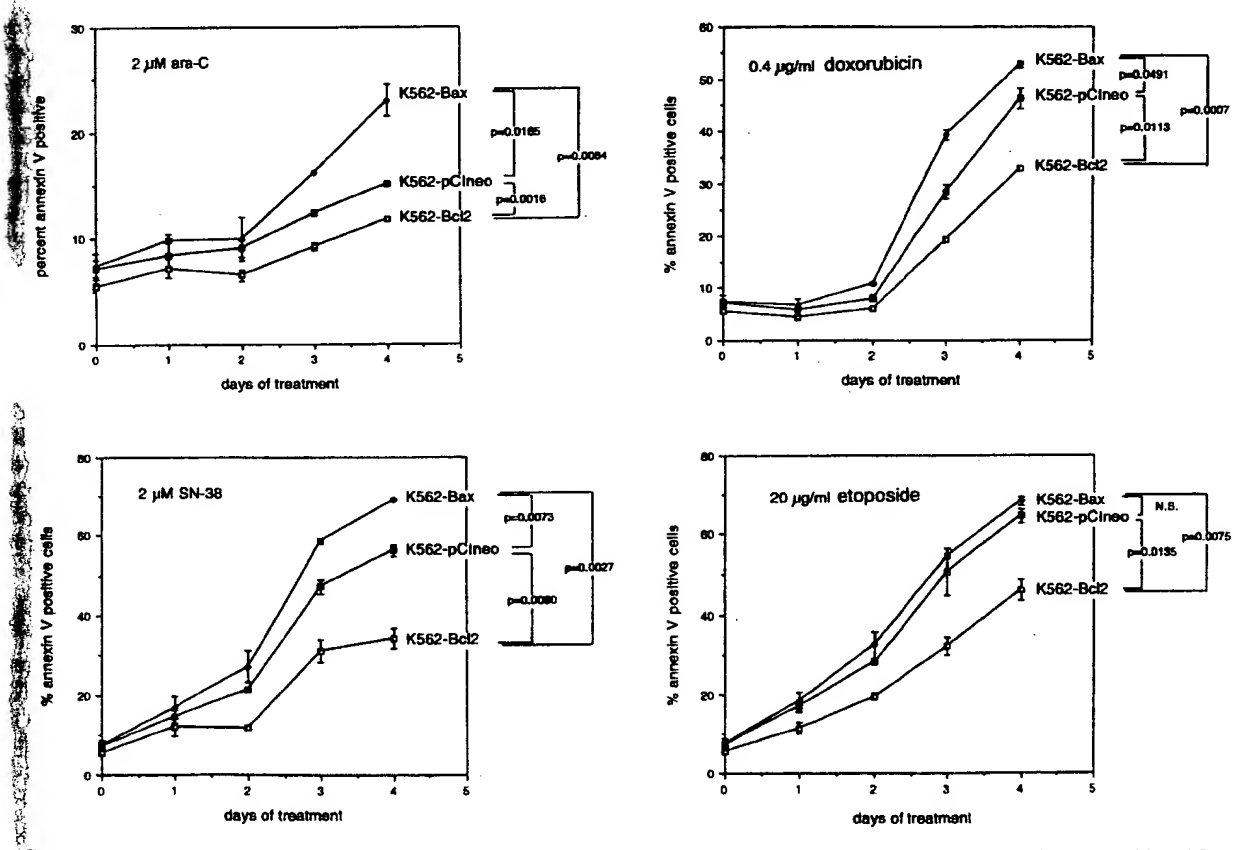

Figure 3 Bax sensitizes K562 cells to apoptosis induced by ara-C, doxorubicin and SN-38, but not by etoposide. After electroporation with Bax, Bcl-2 or pCI-neo vectors, K562 cells were continuously cultured for more than 4 weeks in the presence of G418 to establish stable cell lines, K562-Bax, K562-Bcl-2 and K562-pCI-neo. These three cell lines were cultured in the presence of chemotherapeutic agents ara-C, doxorubicin, SN-38 or etoposide. At different treatment time, cells were taken and analysed for apoptosis by Annexin V assays. Results from three independent experiments were shown

Bax and Bcl-2 play nontraditional roles in apoptosis regulation in these cells. Reports showed that Bcl-2 indeed can promote apoptosis in some cases and that the apoptosis facilitator Bak can inhibit apoptosis in certain situations (Reed, 1997). Apoptosis is accompanied by a loss of membrane phospholipid integrity, resulting in the externalization of phosphatidylserine to the cell surface. The fluorochrome conjugated protein Annexin V binds to phosphatidylserine on the cell surface, this binding can be detected by flow cytometry, which renders this assay a sensitive method to analyse apoptosis (Koopman *et al.*, 1994; Martin *et al.*, 1995; Boersma *et al.*, 1996; van Engeland *et al.*, 1996). Therefore, the Annexin V assay was the method of choice in the present study. Our analysis using a gene transfer system demonstrated that Bax overexpression can modestly induce apoptosis of K562 cells and can sensitize K562 cells to cell death induced by some chemotherapeutic agents.

In a study using a reverse tetracycline system, it was recently demonstrated that Bax expression alone, without additional death stimulus, can induce apoptosis in Jurkat cells (Xian *et al.*, 1996). Parental Jurkat cells express a very low level of endogenous Bax protein. The K562 cells we used in this study express high levels of endogenous Bax protein; nevertheless, further Bax overexpression induced apoptosis. It thus appears that only when Bax is expressed over a threshold can apoptosis be induced, and that threshold seems to differ from cell line to cell line.

It was recently reported that Bax overexpression sensitized human ovarian tumor cells to paclitaxel, vincristine and doxorubicin, but not to carboplatin, etoposide and hydroxyurea (Strobel *et al.*, 1996). In the present study, we used ara-C, doxorubicin, etoposide and SN-38. We also found that Bax overexpression failed to sensitize K562 cells to etoposide-induced apoptosis. These findings suggest that the sensitization effect of Bax is not universal but selective. One intriguing aspect is that this selectivity is not necessarily dependent on the chemotherapeutic agent used. For example, two studies reported that Bax does sensitize cells to etoposide-induced apoptosis. Bax antagonized Bcl-X_L during etoposide- and cisplatin-induced cell death in the murine IL-3-dependent cell line FL5.12 (Simonian *et al.*, 1996a), and Bax deficiency promoted resistance in doxorubicin-, etoposide- and cisplatin-induced apoptosis in mouse embryonic fibroblasts derived from *p53* and *bax* knockout mice (McCurrach *et al.*, 1997). However, in those reports, murine cell lines were used. It remains unclear whether Bax overexpression has different effects of the etoposide-induced apoptosis of human and murine cells.

In our experiment, Bax overexpression did not sensitize K562 cells to etoposide. However, Bcl-2 overexpression protected K562 cells from etoposide-induced apoptosis. This result provides an argument that Bax does not counteract every aspect of Bcl-2-regulated apoptosis and that Bax and Bcl-2 have independent mechanisms regulating apoptosis. It is also conceivable that other apoptosis-promoting members of the Bcl-2 family may counteract Bcl-2 in the regulation of etoposide-induced apoptosis. By the same token, protein dimerization among Bcl-2 family members is not the only mechanism through which apoptosis regulation occurs. It has been reported that Bax antagonizes Bcl-X_L during drug-induced cell death, independent of its heterodimerization with Bcl-X_L (Simonian *et al.*, 1996a). A recent report showed that Bax homodimerization is not required for Bax to accelerate chemotherapy-induced cell death (Simonian *et al.*, 1996b). Further study into the regulation of apoptosis induced by different chemotherapeutic agents may help dissect the molecular pathways through which members of the Bcl-2 family communicate to coordinate the cell death process.

Materials and methods

Cell culture

K562 erythroleukemia cell line was obtained from the American Type Culture Collection (Rockville, MD). K562 cells were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum in a 37°C incubator containing 5% CO_2.

DNA transfection

Bax and *Bcl-2* expression vectors were prepared by inserting *Bax* or *Bcl-2* cDNA into the *Eco*RI cloning site of the pCI-neo vector (Promega). Electroporation was used to transfect expression plasmids into K562 cells, under the conditions described previously (Zhang *et al.*, 1994). Four hours after electroporation, cells killed by electroporation were removed by Ficoll. For stable transfection, cells were selected in medium containing 0.8 mg/ml G418 (Life Technologies Inc.) for 4 weeks.

Treatment of stable cell lines with chemotherapeutic agents

Stable cell lines of K562 cells transfected with *Bax*, *Bcl-2* or pCI-neo vector were treated by continuous exposure to 0.4 μg/ml doxorubicin (Ben Venue Laboratories, Inc., Bedford, OH), 2 μM cytosine β-D-arabinofuranoside (ara-C) (Sigma Chemical Co., St. Louis, MO), 20 μg/ml etoposide (Bristol-Myers Squibb Co., Princeton, NJ) or 2 μM SN-38 (active agent of CPT-11, a topoisomerase type I inhibitor) (Yakurt Inc., Tokyo, Japan).

Western blotting

Total cellular protein was extracted and analysed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as described previously (Zhang *et al.*, 1994). After being transferred to an Immobilon membrane (Millipore, Bedford, MA), the proteins were incubated overnight with antibodies against Bcl-2 (Dako Co.), Bax (Krajewski *et al.*, 1994), p53 (Ab-6) (Oncogene Science) and actin (Ab-1) (Oncogene Science). The levels of protein were analysed using the enhanced chemiluminescence system (Amersham Corp.) according to the manufacturer's instructions.

Apoptosis analysed by Annexin V assay

Cell suspensions at a concentration of 4×10^6 were stained with Annexin V-FITC conjugate (Nexins Research, The Netherlands) in calcium-containing binding buffer at a dilution of 1:100. After 10 min of incubation on ice, data were acquired on a FACScan flow cytometer (Becton Dickinson, Santa Cruz, CA) at 488 nm laser excitation and emission acquired at FL1. Data was analysed using the Lysis II Software (Becton Dickinson).

Statistical analysis

P-values were determined by Student's *t* test.

'ledgements
thanks are extended to Ms Kathryn Carnes for her editorial assistance. This work was supported by NIH grants CA55164 (WZ and MA), CA57639 (MA), and CA16672 (MA).

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J Neurosurg 88:99–105, 1998

Transfection of C6 glioma cells with the *bax* gene and increased sensitivity to treatment with cytosine arabinoside

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Object. Genes known to be involved in the regulation of apoptosis include members of the *bcl-2* gene family, such as inhibitors of apoptosis (*bcl-2* and *bcl-xl*) and promoters of apoptosis (*bax*). The authors investigated a potential approach for the treatment of malignant gliomas by using a gene transfection technique to manipulate the level of an intracellular protein involved in the control of apoptosis.

Methods. The authors transfected the murine *bax* gene, which had been cloned into a mammalian expression vector, into the C6 rat glioma cell line. Overexpression of the *bax* gene resulted in a decreased growth rate (average doubling time of 32.96 hours compared with 22.49 hours for untransfected C6, and 23.11 hours for clones transfected with pcDNA3 only), which may be caused, in part, by an increased rate of spontaneous apoptosis (0.77 ± 0.15% compared with 0.42 ± 0.08% for the vector-only transfected C6 cell line; $p = 0.038$, two-tailed Student's t-test). Treatment with 1 μM cytosine arabinoside (ara-C) resulted in significantly more cells undergoing apoptosis in the cell line overexpressing *bax* than in the vector-only control cell line (23.57 ± 2.6% compared with 5.3 ± 0.7% terminal deoxynucleotidyl transferase–mediated biotinylated–deoxyuridine triphosphate nick-end labeling technique–positive cells; $p = 0.007$). Furthermore, measurements of growth curves obtained immediately after treatment with 0.5 μM ara-C demonstrated a prolonged growth arrest of at least 6 days in the cell line overexpressing *bax*.

Conclusions. These results can be used collectively to argue that overexpression of *bax* results in increased sensitivity of C6 cells to ara-C and that increasing *bax* expression may be a useful strategy, in general, for increasing the sensitivity of gliomas to antineoplastic treatments.

KEY WORDS • **apoptosis** • **chemotherapy** • **brain tumor** • ***bcl-2* gene family** • **gene therapy** • **cell cycle** • **rat**

THE prognosis for patients with malignant gliomas remains dismal, a fact that has sustained interest in the development of new therapeutic strategies. Multiple chemotherapeutic agents have been directed at interrupting processes vital for cell cycle progression and/or cellular growth and proliferation, but recently interest in the ability of these agents to induce programmed cell death (apoptotis) in tumors has increased.[18] However, gliomas are resistant to most chemotherapeutic agents. An increased understanding of the genes involved in the regulation of apoptosis has led to the hypothesis that dysregulation of apoptosis underlies tumor development, progression, and chemoresistance[8,19,36] and that therapies directed at altering the levels of expression of these genes may mediate and/or potentiate the effects of commonly used chemotherapeutic agents.[4,10,19,38]

The *bcl-2* gene family is composed of a group of related genes that either promote or prevent apoptosis.[5,25,34] Members of the family include genes such as *bcl-2*, which is antiapoptotic, and *bax*, which is proapoptotic. Another *bcl-2* gene family member, *bcl-x*, produces two transcripts, $bcl\text{-}x_L$ (long), which is antiapoptotic, and $bcl\text{-}x_S$ (short), which is proapoptotic. These various proteins share significant homology and form either hetero- or homodimers. Heterodimerization of BCL-2 and BAX inhibits apoptosis, whereas homodimerization of BAX promotes apoptosis. The ratio of expression of pro- and antiapoptotic genes likely determines whether a cell lives or dies after an insult.[24] Overexpression of *bcl-2* or *bcl-x* protects cells from apoptosis after a number of different insults,[8,10] whereas overexpression of *bax* renders cells more sensitive to apoptosis-inducing stimuli.[4,32,38] Similarly, loss of *bcl-2* or *bcl-x* can result in excessive apoptosis during development, whereas loss of *bax* can prevent apoptosis.[13,15,22]

The molecular characterization of gliomas has revealed a number of abnormalities, such as *p53* mutations, amplification of the epidermal growth factor receptor, and overexpression of *ras*.[2,35,37] However, there are no data supporting the role of mutations or dysregulation of *bcl-2* gene family members in the pathogenesis or progression of these tumors. A recent study of human gliomas noted an association between wild-type *p53* and *bcl-2* overexpression, whereas in most cases gliomas with mutant *p53* did not show *bcl-2* overexpression.[1] The only study directed at screening for *bax* gene mutations was negative.[9] However, gliomas are sensitive to manipulations of the levels of expression of *bcl-2*; several investigators have shown that production of *bcl-2* overexpression in glioma cell lines protects them from different types of apoptosis-

inducing stimuli.[17,23,39] The effect of *bax* overexpression in gliomas has not yet been characterized.

Gene transfection strategies directed at treating gliomas have included overexpressi n of *p53* or *s-Myc*, tumor n crosis factor-α, p21$^{WAF1/CIP1}$, interleukin-1β–converting enzyme, antisense glial fibrillary acidic protein complementary DNA, and viral thymidine kinase.[3,11,16,20,27,29,40] In general, these manipulations have slowed tumor growth, and in one study, adenovirus-mediated transfection of *p53* caused spontaneous apoptosis in three of six tumor lines examined.[11] Initial reports of experiments with *bax* overexpression in breast and ovarian carcinomas have demonstrated dramatic increases in sensitivity to chemotherapeutic agents.[4,32,38]

The rat C6 glioma cell line, like most malignant gliomas encountered clinically, is resistant to DNA-damaging treatments. The C6 glioma cells typically do not undergo apoptosis even after exposure to high doses of cytosine arabinoside (ara-C) or ionizing radiation. Thus, C6 is a reasonable glioma cell line in which to test the hypothesis that overexpression of *bax* alone is sufficient to increase sensitivity to an apoptosis-inducing stimulus. In this report, we describe the effect of *bax* overexpression in C6 cells. Our findings have led us to suggest that manipulation of the relative levels of *bcl-2* family members may be a useful strategy for increasing the sensitivity of gliomas to conventional chemotherapeutic agents.

Materials and Methods

Generation of a Stable Cell Line Overexpressing Murine bax

The murine *bax* gene was cloned into the pcDNA3 mammalian expression vector and transfected into the C6 rat glioma cell line with a cationic lipid reagent as recommended by the manufacturer. Murine *bax* codes for a protein that has more than 98% homology to rat BAX. Transfected cells were grown and selected in the presence of G418 (500 mg/ml) for 10 to 14 days prior to the isolation of individual clones, and 12 clones were expanded. Clones overexpressing BAX were identified by Western blot analysis. Simultaneously, we created lines of C6 cells transfected with the pcDNA3 vector only (C6.pcDNA3). We tested several C6.pcDNA3 clones that have growth characteristics similar to untransfected C6 and selected one for use as a control line (C6.pcDNA3.10) in the experiments described in this report. The C6.pcDNA3.10 line demonstrated the same growth rate and resistance to chemotherapy as the parent C6 cell line. The C6 cells were maintained in standard medium consisting of Dulbecco's modified Eagle's medium with 15% newborn bovine serum, 0.6% neomycin, 2 mM L-glutamine, and penicillin/streptomycin (100 μg/ml). Clones were maintained in the same medium plus G418 (500 mg/ml). All experiments were performed in G418-free medium.

Assessment of Proliferation

We used a modified version of a previously described colorimetric assay to measure growth curves.[31] Cells were plated onto 96-well microplates at a density of 1000 cells per well in 200 μl of medium. Individual plates were used for each time point, with three to five wells plated for each cell type and treatment condition. After treatment, the cells in each plate were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) at intervals ranging from 0 to 48 hours. After fixation, plates were washed with PBS, stained with 1% crystal violet for 4 hours, washed with distilled water, and all wed t air dry. The crystal vi let was solubilized with 200 μl of 1% sodium dodecyl sulfate (SDS) per well. Optical density (OD) was determined with the aid of a microplate reader with absorbance read at 570 nm. The ODs were averaged for each cell type and time point. We verified the accuracy of this assay independently and determined its accurate working range by comparing assessments of cell number made using the colorimetric assay with those made using traditional cell counts.

Determination of Doubling Time

Data obtained from the proliferation assay were fitted with a logarithmic regression ($y = b*m^x$, where y = OD reading and x = time). The coefficients b and m were determined by using a commercially available curve-fitting program. The doubling time was calculated by the following formula: Doubling Time = ln(2)/ln(m).

Quantitative Assessment of Apoptosis

We used two techniques to assess evidence of apoptosis. The presence of chromatin condensation was determined in cells plated on 35-mm plastic petri dishes modified for ultraviolet (UV) illumination; a glass coverslip was glued over a 15-mm diameter hole made in the center of the dish. Cells were fixed in 4% paraformaldehyde, washed with PBS, and stained with *bis*-benzamide, a fluorescent DNA-binding dye. The cells were viewed at 345/460-nm wavelengths with a fluorescent microscope equipped with a UV filter, and the number of cells showing chromatin condensation were counted. Cells that demonstrated several clumps of densely coalesced nuclei were counted as apoptotic, and nuclear fragments that were not associated with cytoplasm, when viewed under phase-contrast light microscopy, were not counted.[33] Three random × 200 fields were counted in this manner. Phase-contrast microscopy was used to determine the total cell count for each field examined, and the percentage of cells demonstrating chromatin condensation was calculated.

Cells were also stained by using terminal deoxynucleotidyl transferase–mediated biotinylated–deoxyuridine triphosphate nick-end labeling (TUNEL). This assay reveals nuclei undergoing DNA fragmentation in a manner specific for apoptosis. Cells were washed with PBS and fixed with 4% paraformaldehyde in PBS for 1 hour, after which they were washed with PBS and blocked for 15 minutes in proteinase K. Prelabeling and labeling were performed with a commercially available TUNEL kit, which was used according to the manufacturer's instructions. FluoroLink Cy3-Streptavidin was added at a dilution of 1:1000 and the samples were incubated in the dark for 20 minutes, washed with PBS, and viewed with the aid of a UV light-equipped microscope.

Western Protein Immunoblotting

Cells that were in the growth phase were washed twice in PBS, scraped, and pelleted. The cell pellet was lysed in RIPA buffer (0.15 M NaCl; 0.01 M Tris, pH 7.5; 1% Triton X-100; 1% Na deoxycholate; 1% SDS; 0.001% ethylenediamine tetraacetic acid) containing the protease inhibitors aprotinin (1:100) and leupeptin (1:100) and incubated on ice for 20 minutes. The cells were centrifuged again and the supernatant was transferred to a fresh microcentrifuge tube. The total protein concentration of each protein lysate was determined by using a modified Bradford reaction. Equal amounts of protein (50 μg) from each lysate were denatured in 2 × Laemmli buffer and loaded on a 15% SDS-polyacrylamide gel for SDS-polyacrylamide gel electrophoresis (PAGE) with appropriate protein standards loaded in one well. After electrophoresis, the proteins were transferred onto Immobilon-P membranes. Each membrane was blocked in nonfat milk (5%) in Tris-buffered saline (20 mM Tris, pH 7.5; 150 mM NaCl) with 0.05% Tween, and blotted with a primary antibody against BAX (#651, 1:500 dilution), followed by an anti–rabbit immunoglobulin G horseradish peroxidase–labeled secondary antibody (1:5000). The reaction was developed by means of a chemiluminescence reagent and exposed on autoradiography film. Bands were digitized and integrated densities were determined by means of commercially available software. The anti-BAX antibody used does not cross react with BCL-2 or BCL-X (S Korsmeyer, personal communication, 1996).

Fluorescence Analysis

A monolayer of cells was fixed in cold 70% ethanol for at least 2 hours. After PBS washes, the cells were treated with RNase A (100 μg/ml in PBS) for 30 minutes at room temperature. Cells were stained with propidium iodide at a final concentration of 25 μg/ml

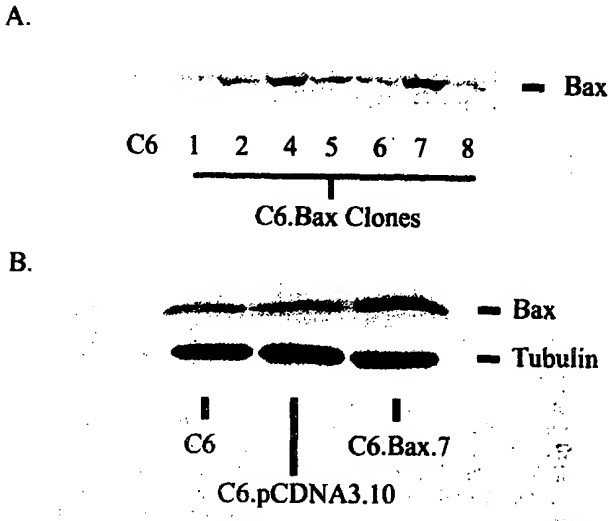

FIG. 1. Western blot gels showing BAX expression. A: Example of a blot used to screen BAX expression in transfected clones. Each lane was loaded with 50 μg of protein. The highest levels of BAX expression were seen in clones C6.Bax.4 and C6.Bax.7. B: Quantitative analysis of BAX expression by untransfected C6 and C6 cell lines transfected with vector only (C6.pCDNA3.10) or with the vector containing the murine *bax* gene (C6.Bax.7). Equivalent amounts of protein (50 μg) were loaded in each lane and the blots were probed for BAX and tubulin. Note the similar expression of tubulin in each cell line and overexpression of *bax* in C6.Bax.7.

for 1 hour. Cell cycle analysis was conducted on a fluorescence-activated cell sorter (FACS) 440 flow cytometer interfaced to a data acquisition/analysis system. The resulting DNA histograms were analyzed for their cell phase distribution with the aid of commercially available software.

Sources of Supplies and Equipment

The pcDNA3 mammalian expression vector was purchased from Invitrogen, Carlsbad, CA, and the cationic lipid reagent (Lipofectamine) and standard medium from Life Technologies, Grand Island, NY. The curve-fitting program (Microsoft Excel) was acquired from Microsoft Corp., Redmond, WA. The *bis*-benzamide (Hoechst 33258) was purchased from Molecular Probes, Eugene, OR. The TUNEL kit was obtained from Trevigen, Gaithersburg, MD, and the FluoroLink Cy3-Streptavidin, protein standards, and ECL chemiluminescence reagent were purchased from Amersham International, Buckinghamshire, England.

The UV light-equipped microscope was purchased from Nikon, Tokyo, Japan. The test kit for the Bradford reaction was purchased from Biorad, Inc., Hercules, CA. The Immobilon-P membranes were acquired from Millipore, Bedford MA. The secondary antibody and autoradiography film were obtained from Caltag, Burlingame, CA, and Eastman Kodak, Rochester, NY, respectively. The FACS 440 flow cytometer was purchased from Becton Dickinson, Mountain View, CA; the CICERO data acquisition/analysis system from Cytomation, Fort Collins, CO; and the MCYCLEAV distribution analysis software from Phoenix Flow Systems, San Diego, CA. Unless otherwise noted, all reagents were purchased from Sigma Chemical Co., St. Louis, MO.

Results

Generation of Clones With Stable Overexpression of BAX

We transfected C6 cells with the full-length murine *bax* gene cloned into the pcDNA3 vector or with the pcDNA3 vector alone. Lysates from 12 independent clones derived from the transfection with the vector containing the *bax* gene were analyzed by SDS-PAGE/Western blotting with an antibody against BAX. A total of five clones demonstrated *bax* overexpression. Figure 1A shows an example of a Western blot test used to screen for BAX expression; two clones (C6.Bax.4 and C6.Bax.7) showed relatively high levels of expression. In Fig. 1B, BAX expression in one of these clones (C6.Bax.7) is compared with that demonstrated by the parent C6 cell line and with a cell line transfected with the vector only (C6.pcDNA3.10). Tubulin expression was used to verify that equal amounts of protein were loaded in each lane. Bands were digitized, analyzed by means of the NIH Image software, and a BAX/tubulin ratio was calculated for each lane (C6:0.30; C6.pcDNA3.10:0.35; C6.Bax.7:0.65). Hence, BAX was expressed at levels more than twofold greater in the C6.Bax.7 cell line than in the parent C6 line. This blot is representative of several blots performed with separate lysates; in all cases BAX expression was at least twofold greater in the transfected cells.

TABLE 1

Doubling times of selected clones of C6 glioma cells

Clone	Doubling Time (hrs)	No. of Wells*
C6 (parent)	22.49	5
C6.pcDNA3.2	22.74	5
C6.pcDNA3.3	24.94	5
C6.pcDNA3.7	22.46	5
C6.pcDNA3.10	23.08	5
C6.Bax.4	32.51	3
C6.Bax.7	33.42	3

* Number of microplate wells tested at each time point.

Overexpression of bax *and Growth Rate of C6 Glioma Cells*

The growth rates of two clones overexpressing BAX (C6.Bax.4 and C6.Bax.7), four vector-only control lines, and the parent C6 line were assessed by means of the microplate growth assays (Fig. 2). Doubling time was determined by fitting a logarithmic function to each growth curve (Table 1). The doubling time for the C6 cell line was 22.49 hours ($r = 0.96$) and for the vector-only control lines it ranged between 22.46 and 24.94 hours (23.31 ± 1.11 hours [mean ± standard deviation {SD}], $r = 0.95$–0.99). The doubling time for two of the cell lines overexpressing *bax* was 32.51 ($r = 0.93$ [C6.Bax.4]) and 33.42 hours ($r = 0.98$ [C6.Bax.7]). Hence, both lines overexpressing the *bax* gene had slower growth rates than the parent C6 and the vector-only control lines, which had similar growth rates.

Cell cycle analysis of the two lines revealed no significant differences in the percentage of cells in the G_1, S, or G_2/M phases (Table 2). Examination of untreated C6.Bax.7 and C6.pcDNA3.10 cells stained using the TUNEL protocol revealed nearly twice as many TUNEL-positive C6.Bax.7 cells as C6.pcDNA3.10 cells (0.77 ± 0.15% compared with 0.42 ± 0.08%, respectively; $p = 0.038$, two-tailed Student's t-test). The similar cell cycle profiles of these two lines together with the increased rate of spon-

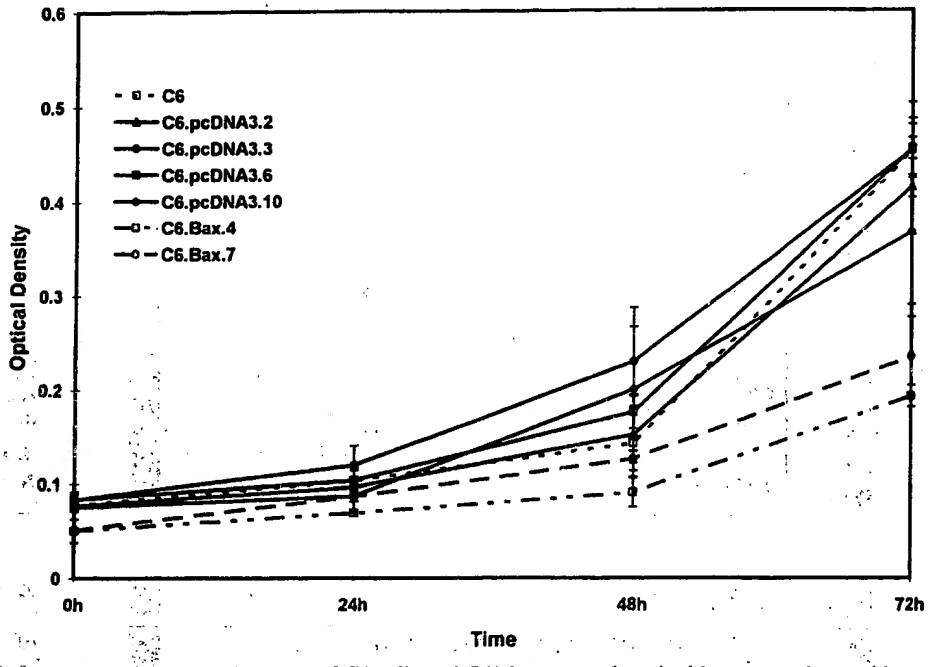

FIG. 2. Graph showing growth curves of C6 cells and C6 clones transfected with vector only or with vector containing the *bax* gene. Individual points were determined by means of a colorimetric assay. Each point represents the mean ± SD of five wells for the C6 and vector-only clones and the mean ± SD of three wells for the clones transfected with *bax*. Zero hour is the first time point for cell measurement, usually 24 hours after plating. Cells were examined in the exponential growth phase. By 72 hours of growth, a distinct difference in growth rates was observed between the clones transfected with *bax* and those transfected with vector only or untransfected.

taneous apoptosis indicate that the difference in doubling time may be caused in part by a higher rate of spontaneous apoptosis in the *bax* transfected cell line.

Sensitivity to Treatment With Ara-C

We treated C6.Bax.7 and C6.pcDNA.10 cells with ara-C for 72 hours at doses ranging from 0.1 to 5 μM and then assayed them for the occurrence of apoptosis with TUNEL staining or by applying morphological criteria after staining with *bis*-benzamide. Treatment of the C6.pcDNA3.10 and C6.Bax.7 cells with 5 μM ara-C resulted in near-total cell loss in both clones, whereas treatment with 0.1 μM ara-C did not induce apoptosis in either clone. Treatment with 0.5 or 1 μM ara-C caused a large percentage of C6.Bax.7 cells to undergo apoptosis. In contrast, the same doses rarely caused apoptosis in C6.pcDNA3.10 cells (Fig. 3). The percentage of cells undergoing apoptosis after treatment with 0.5 or 1 μM ara-C was determined by calculating the fraction of TUNEL-positive cells in each low-power field examined (Fig. 4). These results demonstrate that overexpression of *bax* results in a more than fourfold increase in apoptosis (23.57 ± 2.6% compared with 5.3 ± 0.7% [mean ± SD], $p = 0.007$) after treatment with 1 μM ara-C.

TABLE 2

Cell cycle analysis of vector-only and bax*-transfected C6 cells*

Phase (%)	C6.pcDNA3.10	C6.Bax.7
G_1	54.6	55.3
S	12.3	13.1
G_2/M	33.1	31.6

Impairment of Growth in Glioma Cells Overexpressing BAX

To examine further the effects of ara-C on the proliferation of glioma cells overexpressing BAX, we measured growth curves after 72 hours of treatment with ara-C. Cells were plated, allowed to attach for 24 hours and treated with 0.5 μM ara-C. After 72 hours, the cells were washed with fresh medium to remove the ara-C. The cells were allowed to grow for 1 week, during which time proliferation was measured by means of the microplate assay. Hence, proliferation was assessed prior to treatment (which was used to normalize the assay for each clone), immediately following treatment, and at 48-hour intervals thereafter. Figure 5 shows that the C6.pcDNA3.10 cells continued to grow during treatment with and after washout of ara-C. In contrast, the C6.Bax.7 cells had a prolonged impairment of growth lasting more than 6 days after washout of ara-C. We assayed for the presence of apoptosis in these cells at 72 hours and 6 days after washout of ara-C, but saw only rare TUNEL-positive cells. The FACS analysis of these cells revealed a highly disturbed cell cycle pattern from which no clear picture of G_1 or G_2 arrest could be determined.

FIG. 3. Photomicrographs showing induction of apoptosis in C6 cells transfected with vector only (A and B) or with vector containing the *bax* gene (C and D). Cells were treated with 0.5 μM ara-C for 72 hours. They were stained using the TUNEL protocol (A and C), which reveals only nuclei of cells undergoing apoptosis, or with *bis*-benzamide (B and D), which stains all nuclei but allows for identification of apoptotic nuclei based on morphological criteria. Cells were viewed at × 200 magnification with the aid of a microscope equipped with fluorescent optics. Treatment of C6.pcDNA3.10 with ara-C produces only occasional TUNEL-positive cells (A) or chromatin condensation with *bis*-benzamide (B). Staining reveals most nuclei to be intact; in only the occasional cell is there fragmentation typical of chromatin condensation. C: In contrast, many TUNEL-positive C6.Bax.7 cells are seen after treatment with ara C. D: The same cells as in (C) stained with *bis*-benzamide showing multiple nuclear fragments consistent with the frequent occurrence of chromatin condensation. *Arrowheads* point to examples of chromatin condensation revealed by staining with *bis*-benzamide (B and D).

Discussion

Our study shows that overexpression of *bax* in a rat glioma cell line results in significantly increased sensitivity to treatment with ara-C, which is a DNA-intercalating agent that has been used frequently in clinical chemotherapy protocols. Previous work in our laboratory has shown that untransfected C6 cells do not undergo apoptosis after treatment with even high doses of ara-C. Other investigators have noted that *bax* overexpression in ovarian[32] and breast[4,30,38] carcinomas has resulted in a similar increase in sensitivity to a variety of apoptosis-inducing stimuli. Interestingly, chemotherapy-induced apoptosis after *bax* overexpression does not necessarily require the presence of functional p53,[32] which makes *bax* overexpression an attractive therapeutic modality because up to 50% of malignant gliomas have mutations of *p53*.[2,28,35,37]

The slowing of the growth rate and slight increase in the rate of spontaneous apoptosis of the tumor lines overexpressing *bax* are in contrast to a previous report that *bax* overexpression without any subsequent treatment was insufficient to cause an increase in the rate of spontaneous tumor cell death.[32] However, that conclusion was based on an examination of trypan blue exclusion data only and did not involve direct determination of tumor cell growth rates. In another study, *bax*-transfected cells implanted into SCID mice without any additional treatment showed a reduced rate of tumor formation.[4] Our finding of a small but statistically significant increase in the rate of spontaneous apoptosis in untreated glioma cells overexpressing *bax* (Fig. 4) may be sufficient to explain the difference in growth rates over a long period of time. However, our experimental paradigm did not allow us to examine directly whether *bax* overexpression alone is sufficient to cause apoptosis; we do not think that the small increase in the rate of spontaneous apoptosis we observed is adequate to contradict previous data showing that *bax* overexpression does not cause apoptosis by itself.[38] Furthermore, we do not believe that this small decrease in the growth rate will have significant therapeutic potential by itself. However, our data support the hypothesis that *bax* acts to sensitize cells to apoptosis-inducing stimuli.

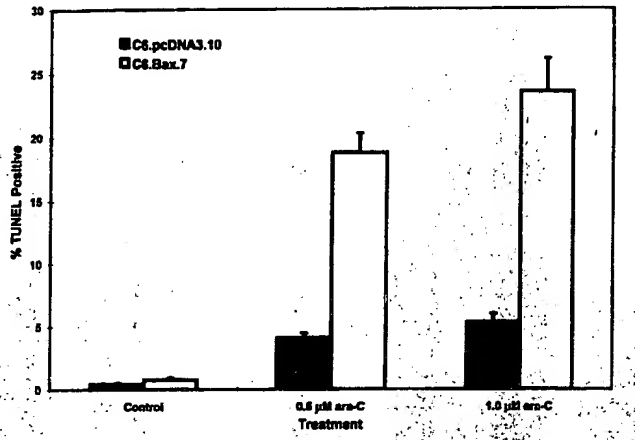

FIG. 4. Bar graph showing quantitative assessment of the prevalence of TUNEL-positive cells following treatment with 0.5 μM or 1 μM ara-C. The percentage of TUNEL-positive cells was determined for three separate high-power fields, and an average ± SD was determined. A significantly increased percentage of TUNEL-positive cells was seen in the C6.Bax.7 cells compared with the C6.pcDNA3.10 line at both doses ($p < 0.008$, two-tailed Student's t-test). A small but statistically significant increase in the percentage of TUNEL-positive cells was also seen in the untreated (control) C6.Bax.7 cells ($p = 0.038$, two-tailed Student's t-test).

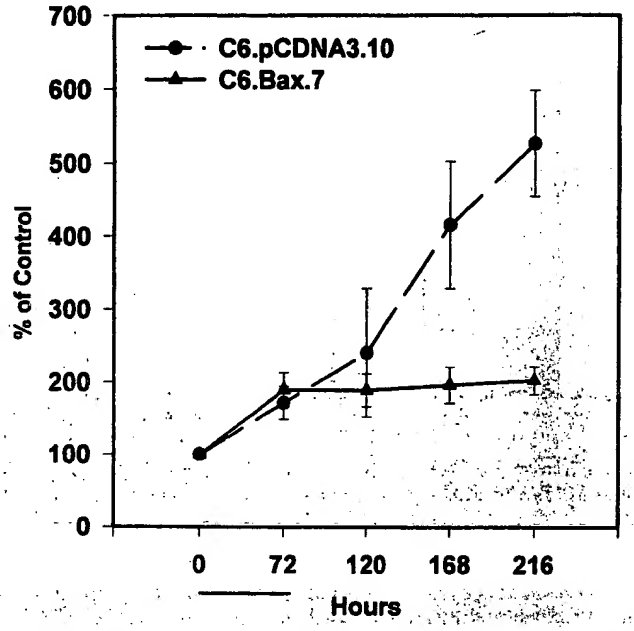

FIG. 5. Graph showing growth curve assay of C6.pcDNA3.10 and C6.Bax.7 cells after treatment with 0.5 μM ara-C. Each time point represents an average ± SD of three wells and is normalized to a point determined at the initiation of treatment (0 hour). Cells were treated with ara-C for 72 hours *(bar under x axis)*, growth was assayed, and the drug was washed out and replaced with normal medium. Growth was determined at 48-hour intervals thereafter. Growth of the C6.Bax.7 cells was severely impaired over the 6 days after washout of the drug, despite regular replacement with fresh medium.

Treatment of glioma cells overexpressing *bax* resulted in a profound disruption of growth that lasted for many days. We did not observe a large increase in spontaneous apoptosis during this time interval, which was one possible hypothesis to account for the lack of growth after treatment with ara-C had ended. Our attempts to characterize the cell cycle by means of FACS analysis during this time interval revealed a highly disturbed pattern that could not be easily defined because statistically significant peaks were absent. Initial reports characterizing the effects of members of the *bcl-2* gene family on the cell cycle indicate that overexpression of *bcl-2* tends to slow the cell cycle[6,26] and overexpression of *bax* tends to speed it up or reverse the cell cycle–slowing effect of *bcl-2*.[6,7] A more detailed analysis of the effect of protracted treatment with ara-C on glioma cells overexpressing *bax* is in progress.

Many new antitumoral therapies have focused on mechanisms of inducing apoptosis. Strategies have included increasing the expression of functional p53, overexpression of tumor necrosis factor-α, overexpression of $p21^{WAF1/CIP1}$, and overexpression of interleukin-1β converting enzyme.[3,11,16,20,40] Protein kinase C inhibitors also cause apoptosis in gliomas.[12,14] The importance of apoptosis in mediating cell death from a potential antitumoral therapy in gliomas was noted by Weller, et al.,[39] who showed that overexpression of *bcl-2* prevented Fas/APO-1 antibody–mediated apoptosis. Also, manipulation of *p53* expression may serve to increase *bax* expression because p53 is a transcriptional activator of *bax*.[21] Hence, activation of specific signal transduction cascades or DNA transcriptional elements may result in altered levels of expression of members of the *bcl-2* gene family, which may enhance sensitivity to apoptosis-inducing stimuli, which include chemotherapeutic agents and ionizing radiation. However, the association between the level of expression of members of the *bcl-2* gene family and response to therapy or outcome in gliomas remains to be determined.

Conclusions

In summary, overexpression of *bax* in the rat glioma cell line C6 results in slowing of the rate of growth and a dramatic increase in sensitivity to ara-C. These findings demonstrate that increasing the level of BAX in C6 glioma cells results in an increased sensitivity to apoptosis-inducing agents and indicate that direct manipulation of the level of expression of members of the *bcl-2* gene family may be a useful strategy for the treatment of primary brain tumors.

Acknowledgments

We thank Dr. Stanley Korsmeyer for kindly providing the murine *bax* gene and anti-BAX antibody. We also thank Robert T. Geist and Dr. Susan Saporito-Irwin for their suggestions and assistance with the cloning procedures, and Dr. Ralph G. Dacey and Patricia Osborne for reviewing the manuscript.

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Manuscript received May 9, 1997.
Accepted in final form August 1, 1997.
This work was supported by Grant No. NS29477 from the National Institutes of Health.

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Recombinant adenoviruses with large deletions generated by Cre-mediated excision exhibit different biological properties compared with first-generation vectors in vitro and in vivo.

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Journal of virology (UNITED STATES) Dec 1996, 70 (12) p8944-60, ISSN 0022-538X Journal Code: KCV
Contract/Grant No.: DK49022, DK, NIDDK
Languages: ENGLISH
Document type: JOURNAL ARTICLE

In vivo gene transfer of recombinant E1-deficient **adenoviruses** results in early and late viral gene expression that elicits a host immune response, limiting the duration of transgene expression and the use of **adenoviruses** for gene therapy. The prokaryotic **Cre - lox** P recombination system was adapted to generate recombinant **adenoviruses** with extended deletions in the viral genome (referred to here as deleted viruses) in order to minimize expression of immunogenic and/or cytotoxic viral proteins. As an example, an **adenovirus** with a 25-kb deletion that lacked E1, E2, E3, and late gene expression with viral titers similar to those achieved with first-generation vectors and less than 0.5% contamination with E1-deficient virus was produced. Gene transfer was similar in HeLa cells, mouse hepatoma cells, and primary mouse hepatocytes in vitro and in vivo as determined by measuring reporter gene expression and DNA transfer. However, transgene expression and deleted viral DNA concentrations were not stable and declined to undetectable levels much more rapidly than those found for first-generation vectors. Intravenous administration of deleted vectors in mice resulted in no hepatocellular injury relative to that seen with first-generation vectors. The mechanism for stability of first-generation **adenovirus** vectors (E1a deleted) appeared to be linked in part to their ability to replicate in transduced cells in vivo and in vitro. Furthermore, the deleted vectors were stabilized in the presence of undeleted first-generation **adenovirus** vectors. These results have important consequences for the development of these and other nonintegrating vectors for gene therapy.

also → Massie (1998) J Vir. 72(3) 2289

JOURNAL OF VIROLOGY. Aug. 1995, p. 4600–4606
0022-538X/95/$04.00+0

Vol. 69, No. 8

Site-Specific Recombination Mediated by an Adenovirus Vector Expressing the Cre Recombinase Protein: a Molecular Switch for Control of Gene Expression

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Received 7 March 1995/Accepted 27 April 1995

We have constructed replication-defective human adenovirus (Ad) type 5 vectors containing the gene for the Cre recombinase from bacteriophage P1 under control of the human cytomegalovirus immediate-early promoter (AdCre). Expression of the protein was detected in replication-permissive (293) and in nonpermissive (MRC5) cell lines, and its biochemical activity was demonstrated in a cell-free recombination assay using a plasmid containing two *loxP* sites. To study Cre-mediated recombination in an intracellular system, we constructed an Ad vector (AdMA19) containing the luciferase cDNA under control of the human cytomegalovirus promoter but separated from it by an extraneous spacer sequence flanked by *loxP* sites which blocked luciferase expression. Upon coinfection of 293 or MRC5 cells with AdMA19 and AdCre, luciferase expression was specifically induced by Cre-mediated excision of the intervening sequence. The use of Ad vectors combined with the Cre-*loxP* system for regulation of gene expression and other possible applications is discussed.

Site-specific recombination systems have recently gained attention for the control of gene expression in eukaryotic cells (40–42) and in animals (3, 18, 19, 27, 31, 33, 39). Extensive use has been made of the Cre-*loxP* system of bacteriophage P1, which requires only two well-characterized components: the 38-kDa recombinase protein, Cre, and the 34-bp *loxP* target sequence (1, 22, 23, 25). Cre binds to the two 13-bp inverted repeats of *loxP* and catalyzes precise recombination between the asymmetric 8-bp core regions of two *loxP* sites (24, 25). Recombination between two parallel sites, as defined by the core region, results in excision of intervening sequences, producing two recombination products each containing one *loxP* site (2, 25), whereas recombination between antiparallel sites inverts the bracketed fragment. Intermolecular recombination between *loxP* sites on separate plasmids results in integration of sequences bracketed by *loxP* sites. The Cre-*loxP* system has been shown to function in both bacteria and eukaryotic cells (2, 25, 38, 40–42, 44) and has been exploited for the excision (19, 27, 31, 38, 40, 41) and the integration of fragments in cellular and viral genomes (12, 32, 42, 43). The use of Cre in cell-free systems for construction of recombinant vectors has also been reported (13, 43). Lastly, Cre-*loxP*-based recombination has been used successfully for tissue-specific gene expression or deletion in transgenic mice (3, 10, 18, 27, 31, 33, 39). In the latter cases, the recombinase was delivered by transfection (10, 18, 19) or microinjection (3, 27, 31) of Cre-encoding plasmids into embryonic stem cells or fertilized eggs.

For the applications described above, it would be useful to have methods for the efficient delivery of the Cre recombinase protein to a large number of cells of different origins. Human adenovirus (Ad) vectors could provide such a vehicle, as they have been used extensively for heterologous gene expression in mammalian cells (4, 14, 15) and have attracted considerable attention as potential recombinant vaccines (14, 15, 34) and for use in gene therapy (8, 28, 35, 45, 48). Ad vectors have a number of properties that render them particularly suited for these and other applications. The 36-kbp double-stranded DNA genome is relatively easy to manipulate with recombinant DNA techniques (14), helper-independent vectors can accommodate up to 8 kbp of foreign DNA, depending on the system chosen (5), and Ad virions are physically and genetically stable if the vectors are constructed and propagated appropriately (6). Viruses with a deletion of E1 can be propagated on 293 cells (16) and can infect other human cell lines but are defective for replication. Ad type 5 (Ad5) can be grown to high titers and can infect a wide variety of tissues, such as epithelial and endothelial cells, fibroblasts, stromal cells, and hepatocytes of different species. Moreover, Ad can infect quiescent as well as replicating cells and express proteins therein.

In this report, we describe the construction and use of Ad vectors in which the Cre protein is expressed under control of the human cytomegalovirus (HCMV) immediate-early promoter (AdCre) and provide evidence for the intracellular action of Cre expressed from these vectors. We also demonstrate that the system can be used to induce expression of a reporter gene by coinfection of cells with two Ad vectors, one carrying the luciferase gene (Luc) under the control of a molecular switch that can be turned on by the second vector expressing Cre.

MATERIALS AND METHODS

Construction of recombinant plasmids. Enzymes used for the manipulation of recombinant DNA and molecular weight standards were purchased from Boehringer Mannheim, Inc. (Laval, Quebec, Canada), Pharmacia (Baie d'Urfe, Quebec, Canada), New England Biolabs (Mississauga, Ontario, Canada), GIBCO Laboratories (Grand Island, N.Y.), or Bethesda Research Laboratories (Burlington, Ontario, Canada) and used according to the suppliers' recommendations. Plasmids were constructed by using standard protocols (37). The Magic PCR or Wizard PCR prep kit was used for the isolation of DNA fragments from low- or high-melting-point agarose gels, respectively. Plasmid DNA was prepared by the alkaline lysis method (7) and further purified by CsCl density gradient centrifugation (37) where necessary. Transformation of plasmid DNA into *Escherichia coli* DH5α {*endA1 hsdR17* (r_K^- m_K^-) *supE44 thi-1 recA1 gyrA* (Nal^r) *relA1* Δ(*lacZYA-argF*)*U169* [φ80d*lac*Δ(*lacZ*)*M15*]} was performed by the $CaCl_2$ method (37).

Oligonucleotides were purchased from The Central Facility of the Institute for Molecular Biology and Biotechnology, McMaster University, Hamilton, Ontario, Canada. A synthetical *loxP* site with compatible *Bam*HI sticky ends, and restriction sites for *Eco*RI and *Sca*I, was obtained by annealing equimolar amounts of two single-stranded oligonucleotides: 5′-GAT CCA ATA ACT TCG TAT AGC ATA CAT TAT ACG AAG TTA TAA GTA CTG AAT TCG-3′ and 5′-GAT CCG AAT TCA GTA CTT ATA ACT TCG TAT AAT GTA TGC TAT ACG AAG TTA TTG-3′. (The diagnostic *Sca*I site, next to the *Eco*RI site, is under-

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lined.) The double-stranded oligonucleotide was 5′ phosphorylated by T4 polynucleotide kinase.

The 5′-CTC CAT AGA AGA CAC CGG GA-3′ primer represents the 3′ end of the HCMV immediate-early promoter and was used for sequencing and in PCR amplification. The second primer for the PCR, 5′-AGA GGA TAG AAT GGC GCC GGG CCT T-3′, binds to the luciferase open reading frame (ORF) from nucleotides 49 to 25. The 5′-CGG ATC CG-3′ oligonucleotide was used to link *Bam*HI sites to blunt-ended fragments.

Cells and viruses. Cell culture media and reagents were purchased from GIBCO. 293 cells were used for growth and titration of Ad vectors as described previously (14, 20). MRC5 cells were grown in alpha minimal essential medium supplemented with 100 U of penicillin per ml, 100 μg of streptomycin per ml, 2.5 μg of amphotericin per ml, and 10% fetal bovine serum for cell maintenance or 5% horse serum for infection.

Construction and growth of recombinant viruses. Recombinant viruses were obtained by cotransfection (14, 17) of 293 cells with the appropriate plasmids as indicated in Results. Plaques were isolated after approximately 14 days and expanded in 293 cells. Viral DNA was analyzed by restriction enzyme digestion as described previously (14). All viruses were plaque purified and reanalyzed prior to preparation of large-scale stocks.

Western blots (immunoblots). Proteins were extracted by incubating infected cells with radioimmunoprecipitation assay buffer (30) for 30 min on ice. Solutions were then cleared of DNA by centrifugation, and aliquots of the supernatants were used for protein separation by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis as described by Laemmli (26). Protein transfer from SDS–10% polyacrylamide gels to Millipore Immobilon P polyvinylidene difluoride membranes (Millipore, Mississauga, Ontario, Canada) was performed at 30 V overnight, using a Bio-Rad transblot cell (Bio-Rad Laboratories, Richmond, Calif.). Western blotting was carried out as described by Towbin et al. (46), using a polyclonal Cre-specific antibody (38) at a dilution of 1:2,500 in Tris-buffered skim milk powder (5%) and a mouse anti-rabbit immunoglobulin G coupled to horseradish peroxidase (Pierce, Rockford, Ill.) as the secondary antibody at a dilution of 1:3,333 in Tris-buffered skim milk powder (5%). The horseradish peroxidase reaction was monitored by using the enhanced chemiluminescence reagents for Western blots from Amersham (Oakville, Ontario, Canada) and Kodak XAR5 (Eastman Kodak Company, Rochester, N.Y.) films.

Southern blot analysis. Restricted plasmid DNA was separated on a 0.8% agarose gel and transferred to a Hybond N membrane (Amersham) as described by Sambrook et al. (37). An enhanced chemiluminescence random prime labeling and detection kit (Amersham) was used to label a plasmid DNA probe as recommended by the manufacturer. Hybridization was carried out at 60°C overnight in a Techne HB-1 Hybridiser (Techne Inc., Cambridge, England). High-stringency washes were twice with 1× SSC (0.15 M NaCl, 0.015 M sodium citrate)–0.1% SDS at 60°C for 10 min and twice with 0.5× SSC–0.1% SDS at 60°C for 10 min.

Preparation of cellular extracts for Cre assay. 293 cells (2×10^7) were infected with the indicated viruses at a multiplicity of infection (MOI) ranging from 2.5 to 10 PFU per cell. Eighteen hours postinfection cells were harvested by scraping and centrifuged at 300 × *g* for 10 min at 4°C. Cell pellets were washed once in 500 μl of 20 mM Tris-HCl (pH 7.5)–300 mM NaCl and resuspended in 500 μl of Cre storage buffer (50% glycerol, 20 mM Tris-HCl [pH 7.5], 300 mM NaCl, 1 mM EDTA [pH 7.5]). Extracts were sonicated four times for 30 s, using a Biosonik III sonicator (Bronwill Scientific, Rochester, N.Y.), and cellular debris was removed by centrifugation at 300 × *g* for 10 min at 5°C. The supernatant was removed and used directly for in vitro Cre assays or stored at −20°C.

Cre assay. Assays for Cre function were carried out as described by Abremski and Hoess (1) and Sauer et al. (43), with the following modifications. Fifty-microliter aliquots of cellular extracts, prepared as described above, were mixed with 1 mM phenylmethylsulfonyl fluoride and 1 mM aprotinin. Tris-HCl (pH 7.5), $MgCl_2$, and acetylated bovine serum albumin were added to final concentrations of 50 mM, 10 mM, and 100 μg/ml, respectively. Cre assays were started by addition of plasmid DNA, and the mixtures were incubated for 30 min at 37°C. The reaction was stopped by phenol extraction followed by chloroform extraction and ethanol precipitation. The plasmid DNA was resuspended in 50 μl of Tris-EDTA buffer, and typically 1 μg of plasmid DNA was used for agarose gel electrophoresis. RNase A treatment was performed during digestion of the plasmids with appropriate restriction enzymes. Digestions were terminated by a pronase treatment (0.5 mg/ml) for 5 min at room temperature.

Luciferase assay. Luciferase assays were carried out as described previously (30).

RESULTS

Construction of recombinant adenoviruses expressing the Cre protein. The 3,439-bp *Hin*dIII fragment of pBS185 (42) containing the HCMV immediate-early gene promoter, the *cre* ORF, and the metallothionein-I polyadenylation signal was cloned into the *Hin*dIII site of the shuttle plasmid pΔE1sp1A (5). The resulting plasmids, pMA1 and pMA2 (Fig. 1), contain the left end of the Ad5 genome with the E1 region replaced by the Cre expression cassette in the left-to-right (pMA1) or reverse (pMA2) orientation relative to the Ad genome. These plasmids were cotransfected with pJM17 (29) into 293 cells to obtain the recombinant adenoviruses AdCre1 and AdCre2 by homologous recombination (Fig. 1).

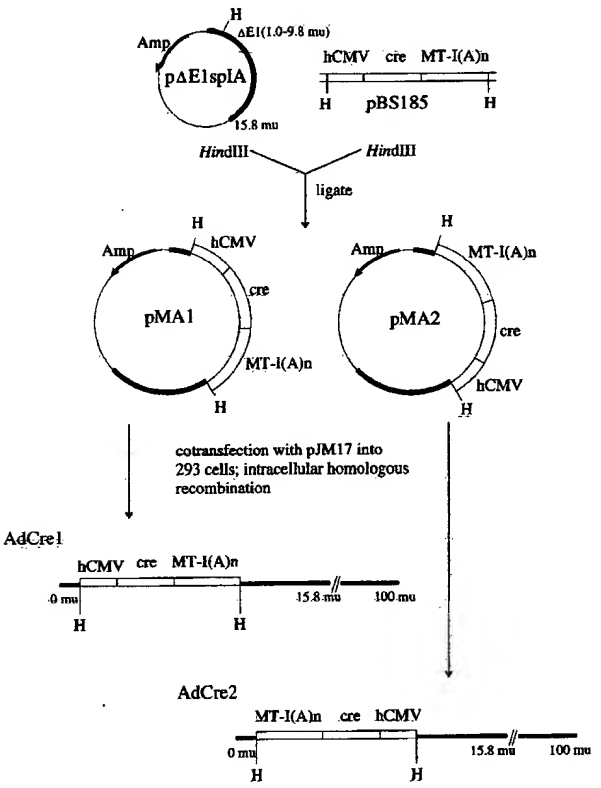

FIG. 1. Construction of Cre-expressing plasmids and viruses. pMA1 and pMA2 were constructed by inserting the *Hin*dIII fragment of pBS185 containing the *cre* ORF (Cre) under control of the HCMV immediate-early promoter (hCMV) and the metallothionein-I polyadenylation signal [MT-I(A)n] into the unique *Hin*dIII site (H) of pΔE1sp1A. AdCre1 and AdCre2 were derived by cotransfection of pMA1 and pMA2, respectively, with pJM17 into 293 cells. Thin lines represent plasmid sequences; solid bars represent Ad sequences. Plasmid sizes and the left end of each virus are drawn approximately to scale. mu, map units.

Expression of Cre from recombinant adenoviruses. Production of the Cre protein by AdCre-infected cells was analyzed by Western blotting with a Cre-specific polyclonal antibody (38). In initial experiments, 293 cells were infected with AdCre1 or wild-type Ad5 at an MOI of 20 or were mock infected and then were harvested at various times postinfection. Expression of the 38-kDa Cre protein was detectable as early as 6 h after infection with AdCre1 and increased up to 24 h (Fig. 2). No further increase was seen at later times (e.g., 36 h), presumably as a result of the onset of cell lysis since 293 cells are permissive for replication of the E1-deficient AdCre1 vector. Cells infected with wild-type Ad5 or mock infected did not express the Cre protein but contained several proteins which appeared to be nonspecifically stained by the polyclonal serum and which were also detected in AdCre1-infected samples. The 60-kDa species, detected only in infected cells, is likely to correspond to the virus fiber protein which is produced at high levels late in infection. Since levels of Cre expression obtained with AdCre1 and AdCre2 did not differ substantially (data not shown), AdCre1 was chosen for further experiments. Expression of Cre protein was also detectable between 24 and 96 h postinfection

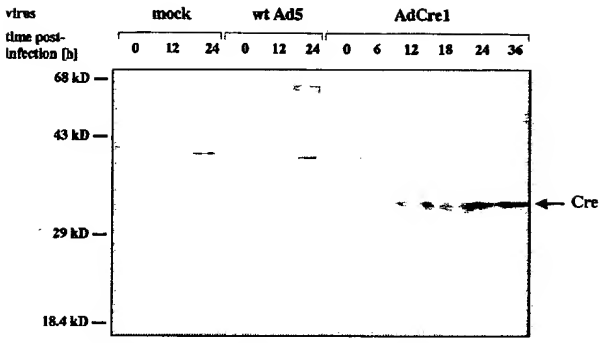

FIG. 2. Detection of Cre expression in 293 cells by Western blot analysis. 293 cells were infected with either AdCre or wild-type (wt) Ad5 at an MOI of 20 or were mock infected and then were harvested for Western blot analysis at the indicated time points. Proteins were separated on an SDS–10% polyacrylamide gel, transferred to an Immobilon membrane, and detected by Cre-specific antibodies. Molecular weights are given on the left, the arrow on the right indicates the position of the 38-kDa Cre protein.

in AdCre1-infected MRC5 cells (MOI of 50) but not in infections with the E1 deletion virus Ad*dl*70-3 (5) at the same MOI or in uninfected MRC5 cells (data not shown).

Recombinant Ad vectors containing the Luc cDNA regulated by a recombination switch. To obtain a protein expression system that could be regulated by Cre-catalyzed recombination, we designed an expression cassette in which the Luc cDNA and the HCMV promoter were separated by a spacer region flanked by *loxP* sites that would prevent luciferase expression unless the spacer was excised. As the recombination product would still contain one *loxP* site between regulatory and coding sequences, we initially investigated whether insertion of *loxP* in this position would allow expression of luciferase. Isolation of such a vector would also confirm that Ad5 could tolerate the palindromic *loxP* sequence in addition to the terminal inverted repeats. Plasmid constructions started with pCA18, which has the E1 region substituted with the HCMV immediate-early promoter and the firefly Luc gene (9) in the left-to-right orientation (2a). A synthetic *loxP* site flanked by *Bam*HI-compatible ends was inserted into the unique *Bam*HI site between the HCMV promoter and the Luc cDNA in such a way that no translational start codon was introduced in any reading frame upstream of the luciferase ATG (Fig. 3A). (If inserted in the opposite orientation, the *loxP* site adds ATGs in two reading frames.) The structure of the resulting plasmid, pMA9, was confirmed by digestion with *Eco*RI and *Sca*I and by sequencing of the insert by using a primer binding in the 3′ region of the HCMV promoter. pMA9 was used with pBHG10 (5) in cotransfection of 293 cells to obtain AdMA9 (Fig. 3A). Our ability to rescue this virus, and its normal growth properties (data not shown), demonstrate that a *loxP* site does not interfere with viral DNA replication.

To generate a construct in which the HCMV promoter is separated from the luciferase ORF by a spacer region, we chose an unrelated sequence with translational start and stop codons in all reading frames that should block luciferase expression. We inserted the 1.3-kbp *Sca*I-*Sma*I fragment of pBS64 (43) into pMA9 that had been linearized by partial digestion with *Sca*I (Fig. 3A). The resulting plasmid, pMA19, contains two *loxP* sites in parallel orientation separated by pBS64 sequences which comprise sequences from pUC12, pBR322, and the SP6 promoter (Fig. 3B). pMA19 was used with pBHG10 to cotransfect 293 cells and obtain the vector AdMA19 (Fig. 3A). AdCA18-3 was obtained by cotransfection of 293 cells with pCA18 and pJM17 (2a). AdMA9, AdMA19,

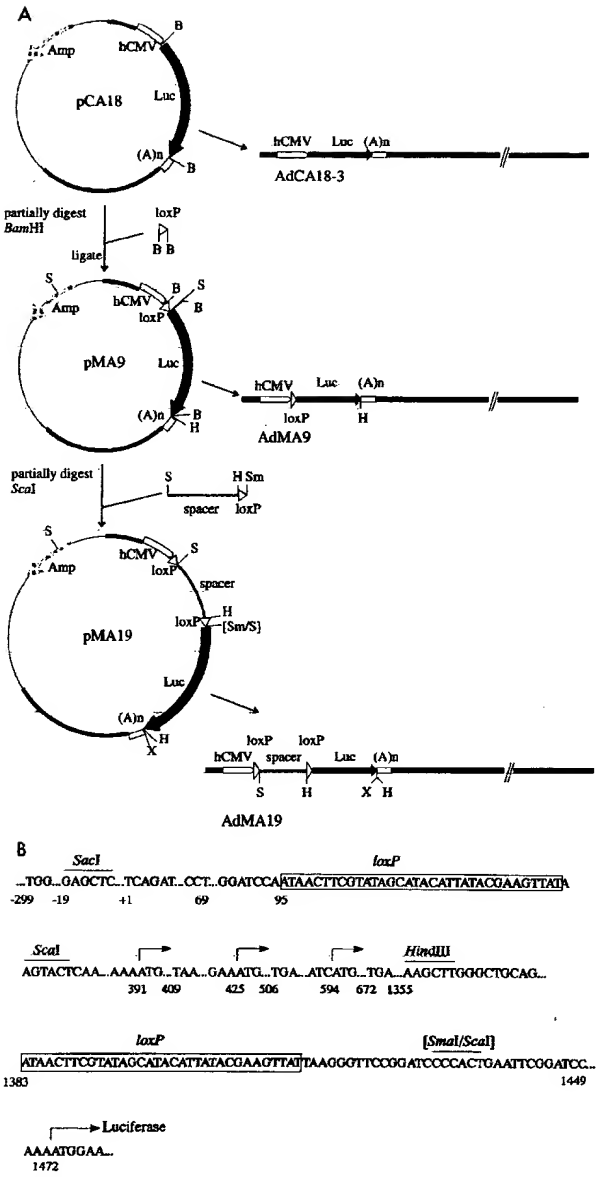

FIG. 3. Construction of regulated reporter plasmids and viruses. (A) pCA18 contains the Luc cDNA under control of the HCMV immediate-early promoter. (A)n designates the SV40 polyadenylation signal. A synthetic *loxP* site was introduced between the promoter and the Luc ORF of pCA18 so that there is no ATG between promoter and Luc cDNA, resulting in pMA9. The spacer region inserted to obtain pMA19 consists of a 1.3-kb plasmid DNA fragment from pBS64 containing a *loxP* site, preceded by start and stop codons in all reading frames. The recombinant Ad vector AdCA18-3 was obtained by cotransfection of pCA18 with pJM17 into 293 cells (2a). To obtain AdMA9 and AdMA19, pMA9 and pMA19, respectively, were cotransfected with pBHG10 (5) into 293 cells. Thin lines represent plasmid sequences; solid bars represent Ad sequences. The left ends of the resulting Ad vectors are shown in detail. B, *Bam*HI; H, *Hin*dIII; S, *Sca*I; Sm, *Sma*I; X, *Xho*I. Plasmid sizes are approximately drawn to scale. (B) Detail of the *loxP*-flanked spacer sequence inserted between promoter and Luc cDNA in pMA19 and AdMA19. Numbering begins at the transcription start (+1) from the HCMV promoter (11). Arrows indicate the luciferase translation start and possible translation starts in all reading frames in the spacer derived from pBS64. Only the first ATG in each reading frame preceded by a purine at the −3 position and the corresponding stop codon in the same frame are shown. Relevant restriction sites are given. *loxP* sequences are boxed.

and AdCA18-3 were further plaque purified before viral stocks were prepared as described previously (20).

Biochemical activity of Cre protein expressed from AdCre1. The biochemical activity of the Ad-encoded Cre protein was first determined in a cell-free assay. Uninfected 293 cells or 293 cells infected with AdCre1 or Add*l*70-3 at an MOI of 10 were harvested 18 h postinfection, and cell extracts were prepared by sonication as described in Materials and Methods. Aliquots of 50 μl were incubated with assay buffer and 1 μg of pMA9 or pMA19 at 37°C for 30 min. After phenol and chloroform extractions, samples were digested with *Hin*dIII and analyzed on an 0.8% agarose gel. *Hin*dIII digestion of pMA19 DNA that had been incubated with extracts from mock- or Add*l*70-3-infected cells generated a 7.9-kbp band and a 1.8-kbp band (Fig. 4). In contrast, when pMA19 was first incubated with extracts from AdCre1-infected 293 cells, *Hin*dIII digestion resulted in two novel bands of 8.4 and 1.3 kbp (Fig. 4B). These sizes correspond to those expected for the linearized forms of the two circular products of recombination: pMA9′ (identical to pMA9 except for 18 bp due to the cloning procedure) and a second corresponding to the excised spacer (Fig. 4A). *Hin*dIII digestion of the control plasmid pMA9 yielded a single linear fragment of 8.4 kbp, irrespective of the extract used for incubation (Fig. 4), since pMA9 contains only one *loxP* site, which does not allow intramolecular recombination. We estimated the Cre-specific efficiency of recombination to be approximately 50%, as judged by the relative intensities of the bands in Fig. 4B. The fragment of ≈11 kbp present only in the Cre-treated sample of pMA19 may represent a Holliday structure (χ) formed as an intermediate of recombination. χ and α structures, derived from Cre mutants, have been observed previously (21) as bands migrating with reduced electrophoretic mobility relative to the unrecombined, digested form of the plasmid. It is possible that sonication or storage of the extract had functionally altered the Cre enzyme, but production of a protein with altered activity in AdCre1-infected cells cannot be ruled out.

Cre-dependent induction of luciferase expression. The expression of firefly luciferase from the recombinant Ad vectors AdCA18-3, AdMA9, and AdMA19 was measured biochemically by emission of light as described previously (30). Infection of 293 cells with AdCA18-3 (no *loxP*) or AdMA9 (one *loxP*) at an MOI of 5 and preparation of cell extracts 24 h postinfection resulted in a luciferase activity of 0.84 ± 0.14 (AdCA18-3) or 0.61 ± 0.03 (AdMA9) μg/10^6 cells, demonstrating that a single *loxP* site inserted between the HCMV promoter and the translation start for Luc did not significantly alter expression of the gene. In a separate experiment, when 293 cells were infected with AdMA9 at an MOI of 10, luciferase activity of about 1.3 μg/10^6 cells was detected after 24 h (Table 1). In contrast, only a low level of activity (20 ng/10^6 cells) was obtained from AdMA19-infected cells, indicating that expression of luciferase from this virus was effectively suppressed by the spacer DNA interposed between the promoter and ATG of the reporter gene. To assess the ability of Cre protein produced from AdCre1 to act on *loxP*-containing Ad vectors, 293 cells were doubly infected with AdCre1 and AdMA9 or AdCre1 and AdMA19 at various MOIs and harvested after 24 h. AdCre1 had no significant effect on the activity of luciferase expressed from AdMA9 (Table 1). However, upon double infection with AdCre1 and AdMA19, luciferase activity was switched on, and depending on the MOI, levels of activity from 0.6 to 2.9 μg/10^6 cells were obtained (Table 1). Induction of luciferase activity suggested that the *loxP*-flanked spacer, between the HCMV promoter and the luciferase ORF, had been excised.

AdCre1-specific recombination, measured as induction of luciferase expression, was also detected in double infections of

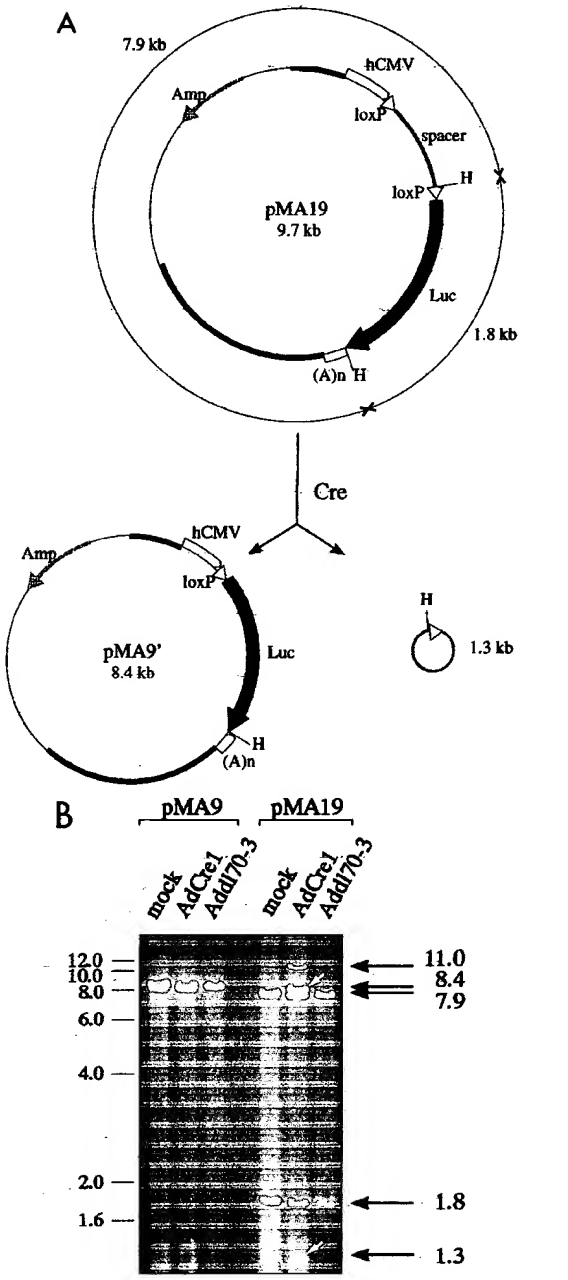

FIG. 4. In vitro recombination assay with pMA9 and pMA19. (A) The expected products after incubation of pMA19 with Cre protein are shown. The product of Cre-mediated recombination of pMA19, indicated as pMA9′, should be identical to the structure of pMA9 except for an additional 12 bp derived from cloning. Open arrowheads represent *loxP* sites. The lightly stippled bar represents the spacer region derived from pBS64, inserted into pMA19 and the expected circular molecule resulting from excision. *Hin*dIII restriction sites (H) and fragment sizes are shown. For further details, see the legend to Fig. 3. (B) One microgram of plasmid DNA was incubated with crude extracts prepared from cells infected 18 h previously with viruses indicated at the top. Molecular sizes (in kilobases) are given on the left margin, and the sizes (in kilobases) of unrecombined and recombined bands are given on the right. Arrows indicate the recombination products of Cre-treated pMA19, as predicted from recombination events shown in panel A.

TABLE 1. Cre-dependent luciferase expression in infected 293 cells

Virus[a] (MOI)	Amt (μg) of luciferase/10^6 cells[b]
None (mock infection)	0
AdCre1 (1)	0
AdCre1 (5)	0
AdCre1 (10)	0
AdMA9 (10)	1.3 ± 0.07
AdCre1 (1) + AdMA9 (10)	1.7 ± 0.4
AdCre1 (5) + AdMA9 (10)	1.2 ± 0.5
AdCre1 (10) + AdMA9 (10)	1.0 ± 0.1
AdMA19 (10)	0.02 ± 0.01
AdCre1 (1) + AdMA19 (10)	0.6 ± 0.2
AdCre1 (5) + AdMA19 (10)	1.8 ± 0.3
AdCre1 (10) + AdMA19 (10)	2.9 ± 0.4

[a] 293 cells were singly or doubly infected with viruses as indicated in a total volume of 200 μl for each 60-mm-diameter dish and harvested 24 h postinfection as described previously (30).

[b] Mean values and standard deviations were obtained from two independent experiments with two dishes for each sample.

the nonpermissive cell line MRC5 (Table 2). No luciferase activity was observed in cells infected with AdCre1 or Add*l*70-3 or when cells were mock infected. As was the case with 293 cells, expression of Cre did not alter the luciferase activity produced by infection with AdMA9, but mixed infections with AdMA19 and AdCre1 increased luciferase expression from a background activity of 2 ng/10^6 cells to 0.6 or 1.6 μg/10^6 cells. Double infections with Add*l*70-3 and AdMA19, which did not result in increased luciferase activity, confirmed that the induction was dependent on the recombining activity of Cre expressed from AdCre1. Although MRC5 cells were infected at a higher MOI than 293 cells and harvested after longer times, they expressed lower luciferase activity than infected 293 cells, even at the highest MOI tested. This finding was consistent with the results of Western blotting, which indicated that Cre was produced at lower amounts in MRC5 than in 293 cells (data not shown).

The background activity of luciferase observed after infection of 293 or MRC5 cells with AdMA19 alone could be due to spontaneous homologous recombination between the *loxP* sites, resulting in excision of the spacer in a small fraction of viruses. To examine this possibility, we performed PCR analyses that would allow detection of recombined viruses. Amplification of parental AdMA19 DNA by using primers binding to the HCMV promoter and the Luc cDNA (see Materials and Methods) should result in a fragment of ≈1.5 kbp, whereas amplification of AdMA19 viral DNA, from which the 1.3-kbp spacer had been excised by spontaneous or Cre-mediated recombination, should yield a 182-bp fragment derived from the excised spacer. In addition to the 1.5-kbp fragment, a faint band of ≈180 bp was detected after amplification of AdMA19 viral DNA (data not shown), in support of our hypothesis that spontaneous excision may have occurred in a small subpopulation of AdMA19 virions.

TABLE 2. Cre-dependent luciferase expression in infected MRC5 cells

Virus[a] (MOI)	Amt (μg) of luciferase/10^6 cells[b]
None (mock infection)	0
AdCre1 (5)	0
AdCre1 (25)	0
AdMA9 (25)	2.3 ± 0.3
AdCre1 (5) + AdMA9 (25)	1.5 ± 0.9
AdCre1 (25) + AdMA9 (25)	2.1 ± 1.0
AdMA19 (25)	0.002 ± 0.001
AdCre1 (5) + AdMA19 (25)	0.6 ± 0.1
AdCre1 (25) + AdMA19 (25)	1.6 ± 0.2
Add*l*70-3 (5)	0
Add*l*70-3 (25)	0
Add*l*70-3 (5) + AdMA9 (25)	1.8
Add*l*70-3 (25) + AdMA9 (25)	2.4 ± 0.03
Add*l*70-3 (5) + AdMA19 (25)	0.003 ± 0.0007
Add*l*70-3 (25) + AdMA19 (25)	0.002 ± 0.0005

[a] MRC5 cells were singly or doubly infected with the viruses as indicated in a total volume of 200 μl for each 60-mm-diameter dish for 30 min at 37°C. Cells were harvested and extracts were prepared 72 h postinfection.

[b] Mean values and standard deviations were obtained from two independent experiments with two dishes for each sample.

Direct demonstration that induction of luciferase activity in cells coinfected with AdMA19 and AdCre1 was due to Cre-specific excision of the 1.3-kbp *loxP*-flanked spacer in AdMA19 was obtained by Southern blot analysis. Viral DNA was extracted 24 h after double infection of 293 cells with AdMA19 and AdCre1 (both at an MOI of 5) and digested with *Hin*dIII. Southern hybridization was carried out by using as a probe the 2.9-kbp *Sca*I-*Xho*I fragment of pMA19 containing Luc cDNA and the spacer segment derived from pBS64 (Fig. 3A). Unrecombined AdMA19 is represented by the 1.9- and 1.8-kbp bands (from the left end of the virus) obtained after double infection of cells with AdMA19 and Add*l*70-3 or after mock infection (Fig. 5). Upon coinfection with AdCre1, the virus-encoded enzyme should mediate recombination, resulting in excision of a 1.3-kbp circle from the viral genome (indicated in Fig. 5A as a 1.3-kbp miniplasmid), leaving behind a 2.4 kbp *Hin*dIII fragment. All of the predicted fragments were detected by Southern hybridization (Fig. 5B). From visual comparison of the intensity of the 2.4 kbp band with that of the 1.8- or 1.9-kbp fragment, we estimate that approximately 50% of the AdMA19 viral DNA had undergone recombination under the experimental conditions used (Fig. 5B). Underrepresentation of the 1.3-kbp fragment is due to the fact that the excised miniplasmid does not replicate, in contrast to the viral vector. Thus, the relative intensity of this band does not reflect the efficiency of the recombination process. The faint bands of 3.1 and 3.4 kbp represent viral DNA fragments and are detected because of contamination of the probe with viral DNA sequences from pMA19, from which the probe was derived. Since no unexpected fragments were generated in this in vivo assay, we assume that the Cre protein is produced correctly in AdCre1-infected cells and that the presence of the 11-kbp band, seen in the in vitro assay (Fig. 4B), was likely due to functional alteration of the Cre enzyme for technical reasons.

DISCUSSION

As mentioned in the introduction, delivery of genes by means of Ad-based vectors is highly efficient and applicable to a wide variety of cell types, both in culture and in vivo. Combining the Cre-*loxP* site-specific recombination system with Ad vectors could therefore provide a powerful new tool for inducing DNA rearrangements and regulating gene expression both in cultured cells and in transgenic animals that contain *loxP* sites engineered in their genomes. As a first step in exploring this approach, we have constructed an E1-deleted vector, AdCre1, containing the coding sequences for Cre under the control of the HCMV immediate-early gene promoter, and a second vector, AdMA19, containing a reporter gene whose expression was regulated by Cre-mediated recombination. We were able to demonstrate the production of the Cre protein in two different AdCre1-infected cell lines: replication-permissive 293 cells and nonpermissive MRC5 cells. Functionality of the Cre protein produced in Ad vector-infected cells was demon-

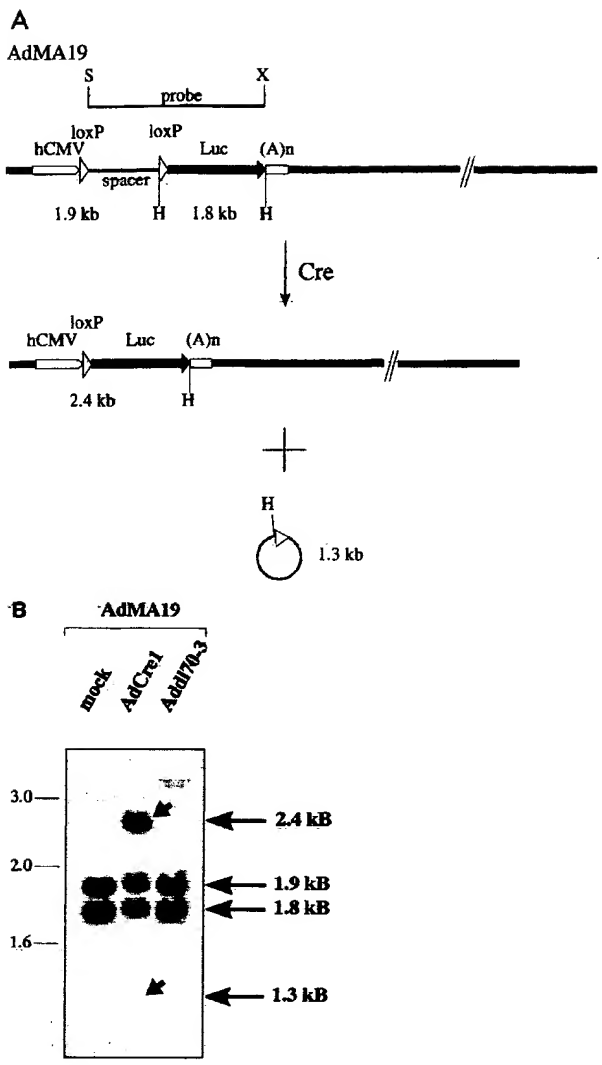

FIG. 5. Detection of Cre-specific recombination of AdMA19 in vivo. (A) The expected recombination products derived from AdMA19 upon coinfection with AdCre1 are shown. Solid bars represent viral sequences, and *loxP* sites are shown as open arrowheads. The spacer sequence blocking luciferase expression is shown as the stippled bar in AdMA19 and as the stippled circle generated by recombination. *Hin*dIII restriction sites (H), approximate sizes of the *Hin*dIII fragments of the left end of AdMA19, the recombined virus, and the excised circle are indicated. (B) Viral DNA extracted from 293 cells doubly infected as indicated at the top was digested with *Hin*dIII. The Southern blot of separated DNA was probed with the 2.9-kb *Sca*I-*Xho*I fragment of pMA19 (see also Fig. 3A). Molecular sizes (in kilobases) are given on the left, and the sizes of fragments derived from unrecombined and recombined viral DNA are indicated on the right. Arrowheads represent the recombination products of AdMA19, as predicted from recombination events shown in panel A. Only relevant restriction sites are shown. For more details, see the legend to Fig. 3.

strated in several assays. Cellular extracts of AdCre1-infected cells specifically mediated recombination of a plasmid containing two *loxP* sites, resulting in precise excision of sequences flanked by them. Double infections with AdCre1 and AdMA19 revealed the recombinatory function of virus-produced Cre protein on a second Ad vector, as excision of the *loxP*-flanked stop sequence was shown by Southern hybridization and, more important, by specific induction of luciferase expression from the recombined AdMA19. The luciferase assays also indicated that the expression of the reporter gene in AdMA19 could be efficiently blocked by the presence of the *loxP*-flanked spacer. We have therefore demonstrated that a system consisting of two Ad vectors, one expressing the Cre protein the other containing a suitably silenced reporter gene, can be used to switch on gene expression in a very specific and efficient manner.

Under the experimental conditions chosen, we estimated that approximately 50% of AdMA19 viral DNA had undergone Cre-dependent recombination. It may be possible to improve on this efficiency by altering the relative MOIs or by staggering the infection times with the two viruses. As the virus-encoded Cre protein contains no nuclear localization signal, addition of this sequence may enhance the efficiency of recombination by increasing the concentration of the protein in the nucleus. It is also possible that immediately after excision of the 1.3-kb DNA spacer from AdMA19, the molecule is efficiently reinserted by Cre (reverse of the reaction diagrammed in Fig. 5A), in which case there may be an upper limit to the fraction of AdMA19 genomes that can be resolved. We detected very low levels of luciferase activity in cells infected with AdMA19 alone and demonstrated that this probably resulted from spontaneous, homologous recombination between the two *loxP* sites in the vector. Consistent with this result, very low levels of Cre-independent recombination in *loxP*-containing molecules have been reported (41). It also has been shown (36) that homologous sequences as short as 14 and 56 bp can be substrates for spontaneous intramolecular recombination of simian virus 40 DNA in mammalian cells, albeit infrequently. If the spontaneous homologous recombination between *loxP* sites should present a problem, its frequency might be lowered by altering the sequence of one of the target sites to reduce the degree of homology, as has been described previously (22, 24).

The system that we have developed might be exploited for the regulated expression of toxic gene products in cells or animals. Cellular or viral genes that currently cannot be expressed at high levels because of the detrimental activities of their protein products could be efficiently delivered into many cells in a silent form and then turned on by infection with AdCre vectors. This system should allow production of toxic proteins at levels sufficient for their characterization and purification. The combination of the Cre-*loxP* and Ad vector systems may also be a particularly useful tool in experiments with transgenic animals in which timed and/or tissue-specific expression of genes is critical. Such studies could include the investigation of developmental processes involving proteins that are suspected of having different functions in embryogenesis and in the adult and that are lethal if expressed in the embryo. Providing Cre protein by a recombinant Ad vector might also prove an easier and more efficient method for the characterization of transgenic mice with Cre-regulated genes than crosses with Cre-containing strains. Lastly, infection with AdCre could be used to knock out genes or gene fragments by site-specific excision of sequences previously flanked by *loxP* sites. This method would allow characterization of proteins with multiple functions and, more important, may overcome the lethality associated with complete knockout mutations since the knockout could be appropriately timed and could be induced in specific organs.

In contrast to retroviruses and adeno-associated virus, which have been used or are being considered for gene therapy, adenoviruses integrate only rarely into the genomes of eukaryotic cells. There is evidence that Ad DNA can persist as extrachromosomal molecules (45), but nonreplicating Ad genomes should eventually be lost from dividing cells. We are interested in exploring whether site-specific integration sys-

tems can be used to increase integration of viral DNA into the genomes of transduced cells. Toward this end, we have successfully used the AdCre1 vector for the site-specific integration of a *loxP*-containing plasmid into the genomes of cultured human cells containing an engineered *loxP* site (47). AdCre may be similarly exploited to target integration of whole Ad genomes or of genes delivered by Ad vectors into chromosomal *loxP* sites of target cells.

ACKNOWLEDGMENTS

This work was supported by grants from the National Institutes of Health, the Natural Sciences and Engineering Research Council, the Medical Research Council, and the National Cancer Institute of Canada. F.L.G. is a Terry Fox Research Scientist of the National Cancer Research Institute.

We thank C. Addison for providing pCA18 and AdCA18-3, B. Sauer for providing pBS64, pBS185, and the Cre-specific antibody, J. Rudy for excellent technical assistance, M. Rudnicki for helpful comments, and S. Bacchetti for critically reading and revising the manuscript.

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